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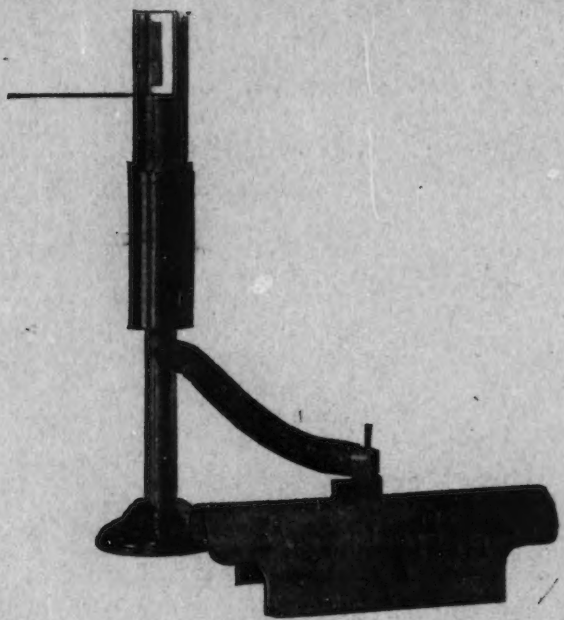

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No. 3. November, 1922

ERRATUM

By an oversight in proof-reading, the name of the joint author of the article on "The Isolation of a Substance from Urine Having Properties of Citric Acid" etc., *page 564 of Volume LX*, was misspelled. The name should read "Mary E. Maver."

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VOL. 62 SEPTEMBER 1, 1922 No. 1

THE CIRCULATION IN THE MAMMALIAN BONE-MARROW[1]

With Especial Reference to the Factors Concerned in the Movement of Red Blood-Cells from the Bone-Marrow into the Circulating Blood as Disclosed by Perfusion of the Tibia of the Dog and by Injections of the Bone-Marrow in the Rabbit and Cat

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From the Laboratories of Physiology of the Harvard Medical School, Boston

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[1] The expense of this research has been in part borne by a grant from the Bache Fund of the National Academy of Sciences. The expense of publication has been in part defrayed by a grant from the Proctor Fund of the Harvard Medical School for the Study of Chronic Disease.

In 1915 and 1916 the possibility of perfusing the tibia of the dog was demonstrated. A description of the original method (1) of perfusion, together with details of the anatomy of the tibia, has been published. These published experiments demonstrated certain facts which made it worth while to refine the methods used and to attack the problem of the mechanism of cell delivery from the marrow tissue to the blood stream.

When oxygenated physiological salt solution was used as a perfusing fluid, early experiments demonstrated: *a*, a very efficient vasomotor control of the marrow vessels—constrictor in type and probably accomplished in the small arterioles within the marrow pulp; *b*, that vasoconstriction brought about by adrenin resulted in a reduction in the number of nucleated red corpuscles and myelocytes found in the effluent from the marrow. These observations led to the conception that the vasomotor control of the marrow vessels might have important bearing upon the extrusion of blood-cells from the marrow.

Experiences with the simple method of perfusion used in the work (1) referred to above led to the endeavor to isolate the tibia completely and to institute an artificial circulation through this bone, employing hirudinized blood from the same animal, avoiding any cessation of circulation, and providing conditions of pulse-pressure, arterial pressure, oxygenation and temperature comparable to those existing before the establishment of the artificial circulation.

I. Method of perfusion of the tibia of the dog: *1. The gross distribution of the blood-vessels of the tibia.* The blood-vessels supplying the tibia may be divided rather artificially into three groups: *1*, minute periosteal arteries springing from fascial and muscular twigs which pass near the bone; *2*, a number of moderate-sized vessels which

enter the extremities of the bone through small foramina, usually in the line of attachment of the joint capsules—arteries which supply the membranes of the joint and frequently terminate inside the bone as marrow vessels; *3*, the nutrient artery.

In considering the blood-vessels of the long bones, one must keep in mind the fact that the circulation, which in early life ministers to bone growth and to blood-cell development, in later life is concerned very largely with the needs of blood formation. In the tibia of a young animal there may thus be little or no intra-osseal connection between the vessels of the epiphyses and those of the diaphysis. When, in the adult animal, the cartilaginous plates separating the epiphyses from the diaphysis have been absorbed, there is a variable degree of anastomosis between the diaphyseal and epiphyseal vessels, and the arteries of class 2 may be considered as directly in series with the nutrient artery, and concerned with the vascularization of the blood-forming marrow rather than with the growth and maintenance of the bone. The degree to which this anastomosis exists varies markedly in different animals but in older animals is never entirely absent. (For a description of the situation in human beings see (2).) The importance of the existence of communication between the nutrient artery which supplies the diaphysis and the epiphyseal circulation is concerned solely with conditions in the lower extremity of the tibia in our perfusions, since the circulation to the upper epiphysis is to a large degree left intact by the method of isolation used. The red marrow of the lower extremity of the bone has been thoroughly circulated in all of our successful perfusions upon adult animals, with the exception of one instance in which an old healed fracture of the lower third of the tibia was present. No twigs of the nutrient artery had crossed the region of this fracture, and the marrow of the lower extremity received its blood supply from the periosteal vessels and from vessels entering the epiphysis around the line of attachment of the joint capsule.

The disposition of the red marrow in long bones has never been thoroughly commented upon. The adult tibia of the dog shows cancellous bone in the upper and lower thirds with a relatively short length of rough-walled cavity between. The actual amount of cancellous bone varies with the amount of blood formation in progress. Animals that have been bled a number of times show a diminution of the cancellous bone and a relatively smooth-walled cavity filled with marrow. Blood-cell bearing marrow shows a constant tendency to concentrate near the endosteum, the center of the medullary cavity along the line of the main

arterial stems showing a larger amount of fat. It is possible that this tendency is due to the fact that in this region there are the capillaries from the perforating periosteal vessels as well as from the nutrient artery, and that the proximity of the endosteum is a causative factor.

There is no method of dissection or preparation of the tibia for perfusion which can make use of the periosteal vessels of class 1 since, at their origin, they communicate freely with muscular and fascial vessels about the bone and thus, if included in the perfusion system, must result in circulation of other than marrow tissue. Langer (3) describes the circulation of bone through the periosteal vessels in the following passage:

> If one cuts a large series of cross-sections of the compact substance of the long bones and these sections are made somewhat thick, one can often find vessels, both arteries and veins, which should be considered as fore-capillaries which pierce the entire thickness of the compact bone. Thus, one finds fairly large arteries which leave the periosteum to anastomose eventually with marrow vessels. The anastomosis occurs without any capillary intervention. It is easy to understand why, in the presence of so many of these vessels, the nutrient artery of long bones is often lacking or very small. They are really nutrient arteries in themselves. Bichat knew of the connection between the outside and the inner arteries of the bones but the final character of this connection is, I believe, still unknown. Bichat succeeded, in a case in which the nutrient artery of the tibia was obstructed, in injecting the marrow of the tibia through the periosteal vessels. I have filled the nutrient artery through the periosteal vessels.

In view of such a description, it would seem that any artificial circulation which neglected these periosteal vessels might result in depriving the marrow of a considerable portion of its normal blood supply. After prolonged observation it has seemed to us that this possible defect in our perfusion technique is not so great as might be anticipated. In the normal adult bone the nutrient artery is undoubtedly the chief source of supply, and its extremely free communication with the periosteal vessels renders it probable that a good circulation can be achieved through it alone. The arteries of class 2 are of great importance. They supply the red marrow of the extremities and anastomose with branches of the nutrient artery. Reference to the diagram (fig. 1) will show three such vessels leaving the popliteal artery near the head of the tibia and passing directly into the bone.

In order to provide direct evidence upon the relations between periosteal and nutrient artery circulation, injections have been made, *a*,

through the nutrient artery alone, and *b*, through the remaining circulation, and in both cases the opposite tibia, injected through the entire circulation, has been used for control.

In the case of the injected bones shown in figures 2 and 3, the following procedures have been carried out:

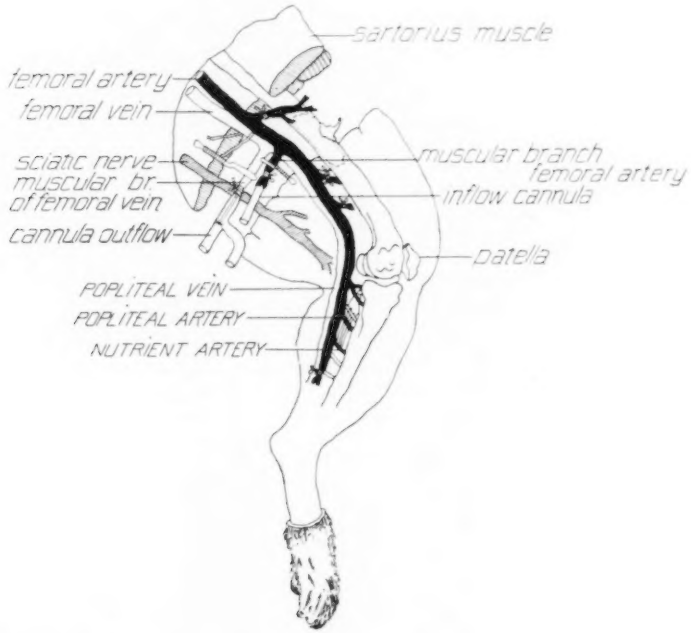

Fig. 1. Diagrammatic representation of the dissection used in the perfusion experiments.

Protocol 1. May 13, 1921. Dog Za, adult female; weight, 15.9 kgm.
8:30 a.m. 30 mgm. morphine sulphate subcutaneously.
9:15 a.m. 20 grams urethane by stomach tube.

The nutrient artery of the left tibia was exposed, adjacent branches of the popliteal artery were ligatured and a cannula pointing centrally was inserted into the popliteal artery below the egress of the nutrient artery. Figure 4, tracing 1, shows the arterial pressure taken through this cannula at 12:10. At 12:35, appropriate connections having been made with the perfusion pump, warm Ringer's solution was driven in through the cannula under the pressure shown in figure 4, tracing 2, and shunted through the nutrient artery and into the tibia by

tying a ligature about the popliteal artery just central to the nutrient artery. A tourniquet was then tightened upon the thigh, and the tibia and fibula removed together from the body. During this operation the perfusion with Ringer's solution was continuous, and, at the end, the bones, stripped of muscle, were placed

Fig. 2. Experiment of May 13, 1921. Injected tibiae of a dog. The bone on the right (left tibia) was injected through the nutrient artery alone; that on the left (right tibia) through the entire circulation. Note that the nutrient artery circulation reaches all parts of the marrow-bearing portion of the bone

upon a pad of moist cotton and warmed from above by an electric light. This operation was completed at 12:41 and Ringer's solution could be seen oozing from periosteal veins over the surface of the bone and from the nutrient vein.

Fig. 3. The tibiae shown in figure 2 split antero-posteriorly in order to show that the vessels injected are not a mere endosteal shell but extend through the marrow.

At 12:44 perfusion was shifted to India ink[2] which was injected from 12:45 to 12:50 under the pressure shown in figure 4, tracing 3. At 12:50 the injection was terminated and the bone in question, prepared by the Spalteholz technique (4), is shown in figures 2 and 3.

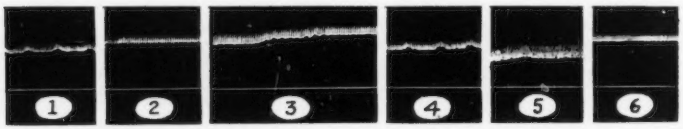

Fig. 4. Blood-pressure relations taken by means of a membrane manometer during the injection of the bones shown in figures 2 and 3.

At 1:45 p.m. a cannula pointing centrally was placed in the left iliac artery and the arterial pressure shown in figure 4, tracing 4, was recorded. Injection with Ringer's solution by means of the perfusion pump under the pressure recorded in figure 4, tracing 5, began at 2:30, the aorta was tied above the iliac bifurcation at the same time, and at 2:32 the injection was shifted to India ink which was introduced for 5 minutes under the pressure shown in figure 4, tracing 6. At the end of this time the right tibia was removed and appears as the bone on the left in figures 2 and 3. Since in this case there was no interference with the circulation to the right leg, this tibia is fully injected but scarcely more so than the left tibia which was injected through the nutrient artery alone.

To secure the bones shown in figure 5, a dog was anesthetized with morphine and urethane, the nutrient artery of the left tibia exposed and a ligature laid around it. A cannula pointing centrally was then inserted in the middle sacral artery through which the blood-pressure shown in figure 6, tracing 1, was taken.

Fig. 6. Blood-pressure relations taken by means of a membrane manometer during the injection of the bones shown in figure 5.

[2] The India ink used in the injection experiments was prepared as follows: To 240 cc. of Higgins' India ink were added 80 cc. of distilled water. This ink suspension was then dialyzed, as recommended by Krogh (5), for varying lengths of time—usually about 90 hours—through a collodion membrane against distilled water in order to free the ink of the traces of preservative present. Before using, the ink suspension was filtered twice through cotton, and when introduced into the animal was at body temperature.

The ink injected at the end of the perfusion experiments was diluted approximately 50 per cent with hirudinized blood, and had not been dialyzed previously.

Perfusion with warm Ringer's solution was then started, the aorta ligatured just above the iliac bifurcation, the ligature tied about the left tibial nutrient artery, and the injection shifted to India ink which was introduced under the pressure relations shown in figure 6, tracings 2 and 3. At the end of 5 minutes the bones were removed and prepared according to the Spalteholz method. They are practically identical in appearance, though in the case of the left tibia the nutrient artery circulation did not exist.

Fig. 5. Injected tibiae of a dog. The bone on the right (left tibia) was injected through the entire circulation with the exception of the nutrient artery which was tied just before injection was begun. The bone on the left (right tibia) was injected through the entire circulation.

These two experiments show clearly how extensive is the communication between the various vessels entering the tibia. In the first case

(fig. 2), the bone, *L*, injected through the nutrient artery alone shows a small area at the lower epiphysis which, though reached in slight measure by the injection mass, remains comparatively free of ink. The same thing, but to a less degree, is seen in the upper epiphysis. In the case of our perfusion experiments, however, the upper epiphyseal circulation has been more perfect since several large vessels to this extremity have remained uncut and, if inefficient flow has existed, it has been in the smaller lower extremity alone.

2. Isolation of the tibia for perfusion. The isolation of the tibia is accomplished as follows: A dog, weighing 8 kgm. or over, is anesthetized with a subcutaneous injection of morphine sulphate, followed by urethane (ethyl carbamate) by stomach tube. This combination gives an ideal anesthesia for protracted experiments. If the animals are kept warm, blood-pressure should remain normal for 12 hours. Artificial respiration has never been necessary in our experiments.

A skin incision in the left leg is made, extending from 1 inch below the groin down the line of the femoral artery, across the knee-joint and to a point $1\frac{1}{2}$ inches above the ankle-joint. This incision is kept anterior to the saphenous vein which should not be cut and tied until near the end of the operation. The femoral artery is dissected out to the knee-joint, *all* branches being securely ligatured with fine silk. Throughout every part of the operation great care must be used in hemostasis since it is of obvious importance that the animal lose no blood during the dissection and that no small vessels exist in a leaky condition when the perfusion with incoagulable hirudinized blood is started.

The dissection which we have carried to the knee-joint is now pushed further, the popliteal artery (continuation of the femoral artery) being cleaned down to the lower limits of the skin incision. All branches are tied with silk except such vessels as can be traced to the bone in the upper extremity and the nutrient artery, which is the lowermost of the arteries shown in the diagram (fig. 1). The nutrient artery, like the other vessels to the tibia, is extremely small and has its origin and course in muscle. In order to find and to preserve it, the operator must observe all the precautions outlined in the article which first described the technique of perfusing the bone-marrow (1).

A ligature is now tied securely about the popliteal artery, 1 inch below the egress of the nutrient artery. It is clear that at this point all the blood flowing down the femoral artery passes into the tibia. There is no other open arterial path. It is now necessary to cut off other

arteries to the lower leg which might make anastomotic connections with the tibial marrow through the periosteal vessels. This is done by cutting through the muscles and skin of the thigh between bulk ties of these structures. Again, careful hemostasis must be maintained. As a final block, the femur is notched into the marrow and the medullary cavity tightly packed with gauze. This prevents any possibility of loss of blood through veins which have eventual communication with those leaving the tibia around the knee-joint. When this section has been finished, the only structures connecting the thigh and leg are the femur, the sciatic nerve, the femoral artery and the femoral vein. The femoral artery is the single path for blood to the leg and our earlier dissection has directed this blood to the tibia alone.

Venous blood may leave the tibia in a variety of ways: *a*, through periosteal veins, *b*, through larger veins passing out from the extremities of the bone, and *c*, through the nutrient vein. But no matter what vein takes blood from the tibia, there is no path across the gap made by our skin and muscular section in the thigh save by the femoral vein, and this fact gives opportunity to collect all the blood flowing through the bone, and to measure the rate of flow and cellular composition of this blood. It is clear that figures for rates of flow obtained in this way do not represent the complete natural flow through the bone. They probably are always somewhat lower than would be found in the undissected part, could such measurements be made. This possible deficiency is due, as has been pointed out, to the impossibility of including any of the perforating periosteal vessels of class 1, and to the necessity of ligating the popliteal artery below the egress of the nutrient artery in order to shunt the perfusing blood into the bone. This latter ligature cuts off the vessels of class 1 and class 2 which normally reach the lower extremity.

The operation, as outlined so far, requires in the neighborhood of 2 hours, and results in the vascular isolation of the tibia. Our next steps are directed toward establishment of an adequate artificial circulation.

3. Establishment of perfusion. It is first essential to determine the pressure conditions in the isolated tibia as circulated by the animal, and also to measure the rate of normal blood flow in the preparation. The femoral artery of the opposite leg is exposed and a cannula is inserted in a side branch at the level of the artery used for perfusion. This cannula is connected through rubber tube *18*, figure 7, with a membrane manometer.

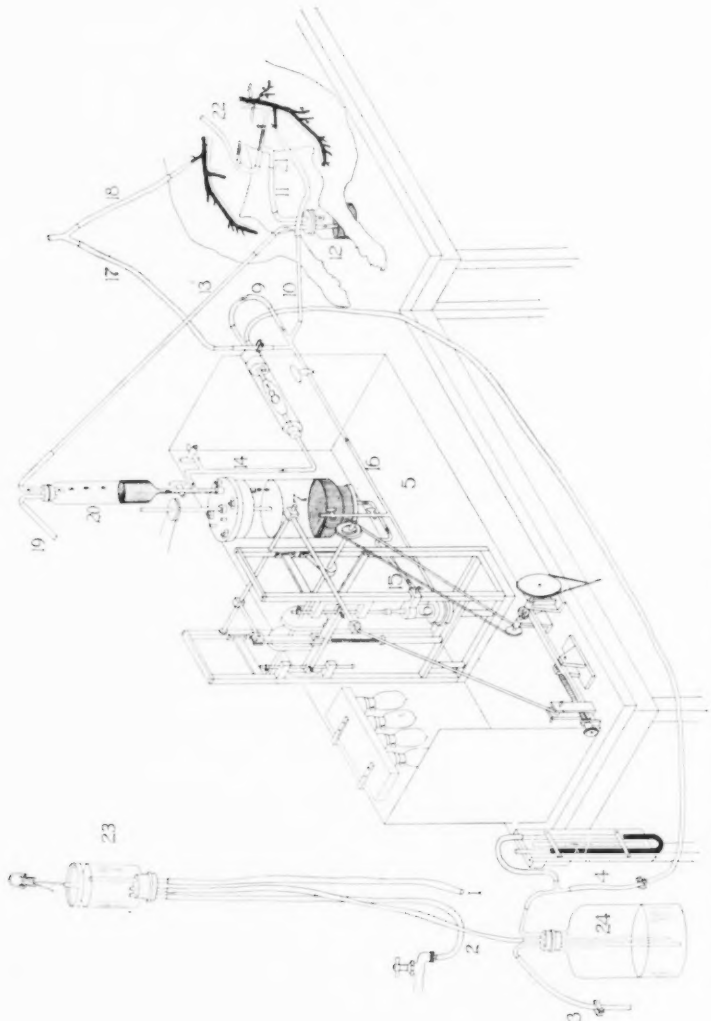

Fig. 7. The perfusion pump with arrangements made for tibial perfusion.

1, Overflow tube from pressure reservoir; *2*, tube to pressure reservoir; *3*, emptying tube; *4*, tube to by-pass resistance chamber; *5*, galvanized water bath; *6*, pump; *7*, artificial lung; *8*, by-pass resistance chamber; *9*, tube leading to resistance chamber; *10*, arterial inflow tube from pump; *11*, venous outflow from bone; *12*, venou reservoir; *13*, return tube to lung reservoir; *14*, by-pass to lung; *15*, pump inflow tube; *16*, pump outflow tube; *17*, pump pressure to membrane manometer; *18*, arterial pressure to membrane manometer; *19*, tube to suction pump; *20*, lung reservoir; *21*, washout cannula; *22*, tube to washout mechanism; *23*, water-pressure reservoir; *24*, pressure bottle.

A washout cannula, *21*, is inserted in the large and constant muscular branch of the femoral vein which accompanies the perfusion artery. This vein has been isolated during the previous dissection, ligatured, and cut $1\frac{1}{2}$ inches from its entrance into the femoral vein. The washout cannula is inserted into the central stump which is seen to join the femoral vein as it crosses the gap between the sectioned muscles of the thigh. (See also fig. 1.) Tube *22* connects this cannula with the washout system. Tube *11*, passing to the venous reservoir, is, as yet, not connected. A tracing is now made, through *18*, which indicates the pressure in the opposite femoral artery at the level of eventual inflow in the leg carrying the tibia to be perfused. As soon as this is completed, measurements of the normal rate of blood flow through the tibia prepared for perfusion are made by means of the washout cannula. This is readily accomplished by moving the bulldog clamp which has blocked this cannula up on to the femoral vein central to the entrance of the vein containing the cannula, thus shunting to a collecting graduate all the blood passing from the part. On shifting the clamp to its original position, blood at once flows up the femoral vein in its normal course, and by use of the washout system the cannula can be kept completely free from obstructive clots. After taking two tracings and several determinations of rate of flow at 5-minute intervals, the animal is bled from the carotid artery into a graduated bottle containing hirudin, and the hirudinized blood run into the separatory funnel, *20*, by removal of the stopper. The stopcock in this funnel being opened, the blood runs down into the artificial lung, *7*, which, with slight modifications, is constructed upon the plan outlined by Hooker (6). It consists of a 1000 cc. glass bottle from which the bottom has been ground off and replaced by a brass collar and cover, the collar cemented in position and the cover carefully fitted so that, after greasing and tightening the wing nuts on the top, the lung is practically air-tight. Through the center of the cover there passes a shaft belted to a small motor above and having upon its lower end a circular glass plate 3 mm. less in diameter than the inside of the bottle. When the motor is turned on, this plate revolves rapidly, and blood dropping upon its surface is thrown against the sides of the bottle in a thin film and runs slowly to the bottom. Small tubes passing through the cover permit the exposure of the blood to oxygen, air or any desired gas mixture. No frothing is encountered, the blood is kept in contact with glass alone and has ample opportunity for gas exchange before it collects in the bottom of the bottle and is drawn into the pump, *6*, through

intake tube *15*. The actual construction of the glass pump, *6*, and the methods available for prompt adjustment of pulse-pressure can be obtained by reference to an article by Richards and Drinker (7), published in 1915.

When the pump is in operation, blood is pumped out through tube *16*, and on getting outside the water bath, *5*, may take two courses—through tube *10* to the arterial cannula in the animal or through tube *9*, which is a constant pressure by-pass through chamber *8* and tube *14* back to lung *7*. The construction of chamber *8* is that used by Knowlton and Starling (8) for the capillary bed in their isolated heart preparation. A rubber tube, made by cutting off the tip of an ordinary finger cot, is tied securely between the ends of tubes connecting with *9* and *14* and is enclosed in a glass cylinder. The cork closing the outer end of this cylinder possesses a second opening which is connected with a long rubber tube, *4*, leading to the constant pressure apparatus at the extreme left of the illustration. The pressure in this apparatus depends upon the height of the water reservoir, *23*, which is fed constantly from the tap and overflows through tube *1* into the sink and through tube *2* into a closed bottle, *24*, from which air pressure is led off to tube *4*. By varying the height of reservoir *23*, it is easy to vary the pressure inside the by-pass chamber, *8*, and as the pressure in this chamber plays upon the finger cot leading blood back to the lung, it is clear that the pressure in the entire system may be varied rapidly or held absolutely constant. The amount of blood passing through the tibia will thus depend upon the condition of the tibial vessels. If they are dilated, then, under any given conditions of pressure provided by the constant pressure by-pass, a certain amount of blood will flow through tube *10* and through the bone. If they are constricted, less blood will pass through the bone and more through the by-pass. The situation provided is exactly that seen in the intact vascular apparatus, where a constriction in one area shunts blood to another. In the case of a tissue in which the blood flow is not great and with the utilization of a pump which possesses practically unlimited power, it is necessary to have a by-pass in order to avoid the development of undue pressures, and, further, when the actual blood in circulation in the tissue represents but a small fraction of that in the system, it is of advantage to have the by-pass in operation, since through its operation the blood is kept in motion and thoroughly stirred.

Blood which passes into the tibia is collected through cannula *21*, and by tube *11* is connected with reservoir *12*. From this reservoir

it is lifted by negative pressure through tube *13* to separatory funnel *20*, from which it passes into the lung and back into general circuit. Tube *19* leads to a negative pressure water pump.

As is obvious from figure 7, the major part of the pump is placed in a large galvanized iron water bath, *5*. This bath is kept at 39° to 40°C. by means of electric lights and a thermoregulator. In order to be sure that the blood entering the bone is at body temperature, the inflow cannula has a side arm, not shown in the diagram, through which a small thermometer is inserted.

The apparatus employed is easy to take apart and clean, and, if need be, can be sterilized. In the case of the experiments to be described, this last refinement has not been attempted. Very thorough cleaning with soap and water, followed by rinsing with hot tap water, preceded each assembly of the pump. After assembly and just before beginning the actual experiment, the inside of all the tubing was cleaned by pumping through it sterile physiological salt solution. Figure 7 indicates that there is a certain amount of rubber tubing used for connections which comes in contact with the circulating blood. In the actual preparation this has been reduced as far as possible by sealing in glass tubes, and, where this is impossible, by bringing the ends of the glass tubes together within the rubber. The best grades of black, pure gum tubing have been employed, and the possibility of untoward effects upon the blood from contact with rubber has been reduced as far as possible. The blood comes in contact with metal at no point in the circuit.

II. The effects of anesthesia and hirudin. In the perfusion and injection experiments to be described, morphine sulphate and urethane were used to provide anesthesia in dogs, and urethane alone was employed for cats and rabbits. In every case it was our desire to obtain correlation between the condition of the bone-marrow and the cellular content of the circulating blood, with particular reference to the manner in which adult red cells reach the circulation. In order to make a direct attack upon this problem, we were compelled to devise a very extensive operation which, of course, required anesthesia. Krumbhaar (9) has shown that in dogs one hour's etherization without operation produced a rise in leucocyte count from 14,000 to 20,000 in one instance, and from 14,000 to 17,000 in another. The rise is far greater if etherization is accompanied by operation, 16,000 to 34,000 at the end of one hour being a characteristic result. Mann (10) in a larger series of observations has shown the same fact for ether alone,

and has emphasized the variability in the extent of the reaction. King (11) has followed the course of non-septic leucocytosis in patients operated under chloroform anesthesia, and, in one instance, records the appearance of nucleated red corpuscles in the circulating blood 30 hours after operation and accompanying a leucocytosis of 20,900. All of these observations, and others which might be added to them, indicate that ether and chloroform cause movement of leucocytes from the bone-marrow to the blood, and Mann (10), by finding a uniform leucocyte content of blood taken from different parts of the body, has confirmed this indication.

In the case of morphine and urethane anesthesia, the leucocytes are affected much as in the case of ether. An instance in which an animal was anesthetized with morphine and urethane but in which no operation was performed is shown in protocol 2. Here, as in all of our experiments, blood counts are expressed in terms of the number of corpuscles per cubic millimeter of blood.

Protocol 2. November 6, 1916. Dog I, female; weight, 7.2 kgm.
12:30 p.m. Leucocytes 16,700; erythrocytes 4,696,000; nucleated red cells 67.
12:33 p.m. 30 mgm. morphine sulphate subcutaneously.
2:40 p.m. 10.8 grams urethane by stomach tube.
2:41 p.m. Leucocytes 18,500; erythrocytes 4,904,000; nucleated red cells 74.
4:40 p.m. Leucocytes 20,200; erythrocytes 4,268,000; nucleated red cells 40.
5:00 p.m. Leucocytes 21,200; erythrocytes 4,680,000, nucleated red cells not counted.

When operation is added to the anesthesia much larger effects may result. These are illustrated by the following protocols:

Protocol 3. February 16, 1918. Dog N. N., male; weight, 19.9 kgm.
8:45 a.m. 75 mgm. morphine sulphate subcutaneously. Leucocytes 18,200; erythrocytes 7,726,000; nucleated red cells 0.
10:25 a.m. 24.9 grams urethane by stomach tube.
10:50 a.m. Operation exposing nutrient artery to tibia begun.
12:45 p.m. Leucocytes 26,700; nucleated red cells 0.
3:15 p.m. Operation on tibia completed and all connections made with perfusion pump. Leucocytes 28,600; erythrocytes 7,385,000; nucleated red cells 29. (In this case, 1 nucleated red cell was seen in counting 1000 leucocytes. As this apparent increase was not verified in later specimens, it may be disregarded.)
Protocol 4. November 3, 1917. Dog J. J., male; weight, 16.1 kgm.
8:30 a.m. 75 mgm. morphine sulphate subcutaneously.
9:15 a.m. 20.1 grams urethane by stomach tube.
9:32 a.m. Leucocytes 13,100; erythrocytes 7,802,000; nucleated red cells 0. Operation exposing nutrient artery to tibia begun.
1:30 p.m. Operation finished.
2:47 p.m. Leucocytes 28,500; erythrocytes 7,447,000; nucleated red cells 19.

These experiments make it clear that the anesthesia and operation necessary for tibial perfusion produce leucocyte migration from the marrow. It has been pointed out elsewhere (12) that movement of cells from the marrow can result from three sets of circumstances: *a*, ameboid migration of cells; *b*, crowding out through growth pressure; and *c*, bone-marrow disintegration. The experiments quoted, and others to which it is unnecessary to give space, show that the effect of anesthesia and operation is largely upon the leucocytic series. Rapid growth of any form of cell in the marrow cavity may cause the appearance of cells of other types in the circulation, as has been noted by Ewing (13), Rieder (14) and Sherrington (15).[3] But mere migration into the blood stream of leucocytes already existing in the marrow does not seem to force out young red forms. When the marrow is intensely hyperplastic, the situation is different. Anesthesia and operation may then be accompanied by a large escape of nucleated red cells, as shown in the following protocols.

Protocol 5. January 11, 1917. Dog T, adult male; weight, 16.5 kgm.

8:50 a.m. 75 mgm. morphine sulphate subcutaneously.

9:20 a.m. 20.6 grams urethane by stomach tube. Leucocytes 19,700; erythrocytes 6,624,000; nucleated red cells 446.

9:40 a.m.–12:45 p.m. Operation isolating tibial circulation.

2:25 p.m. Leucocytes 38,600; erythrocytes 7,184,000; nucleated red cells 1428.

Protocol 6. March 22, 1922. Dog D. D. D., young female; weight 7.2 kgm.

12:40 p.m. Leucocytes 19,800; erythrocytes 7,244,000; nucleated red cells 5474.

12:55 p.m. 18 grams urethane by stomach tube.

1:35 p.m. 5 grams urethane by stomach tube.

2:00–4:45 p.m. Operation isolating tibial circulation.

5:28 p.m. Leucocytes 25,800; erythrocytes 8,841,000; nucleated red cells 8204.

In both of these cases many nucleated red cells were present in the circulation before anesthesia was begun. The condition of the bone-marrow in dog D. D. D. is described in connection with the perfusion of that animal, and a drawing of this marrow appears in figure 18. The blood current is apparently in direct contact with columns of nucleated red cells which have grown into the capillaries, and anesthesia under these circumstances seems to cause the young red cells to lose cohesion with one another. Being unprotected from the moving blood

[3] In our opinion, the observation quoted from King (11) is another case of the same sort.

stream, they are thus slowly washed away and appear in the peripheral circulation. The marrow in the case of dog T is similar in appearance to that of dog D. D. D., and the results of perfusion were the same. The situation presented amounts, then, to this: In the case of the ameboid leucocytes, anesthesia and operation cause migration from the marrow into the blood stream, but the non-ameboid nucleated red cells remain in the marrow, unless at the time of the experiment they are directly in contact with the moving blood current, or unless anesthesia is so protracted as to cause breakdown of the walls of the marrow capillaries and so expose to the blood stream cells in their normal situation outside the vascular bed.

Owing to the necessity of using hirudin to prevent blood coagulation, another factor which disturbs the cellular content of the blood is found in our experiments. The hirudin used was made by E. Sachsse & Co. of Leipzig. Marshall (16) has shown that hirudin supplied by this firm has possible toxic qualities when given intravenously to dogs. The most important effects which he reports consist of prostration, vomiting, diarrhea, and petechial hemorrhages coming on some time after the injection. Experiences like his, while precluding the use of commercial hirudin for such purposes as human transfusion, do not necessarily nullify its use for brief studies upon single organs, as has been amply shown by the work of Starling (8) and his collaborators upon the mammalian heart-lung preparation, and by Richards and Plant (17) on the isolated kidney. There is, however, a much more direct objection to the use of hirudin in studies involving the bone-marrow. Löwit (18) has shown that sodium chloride extracts of leeches cause a leucopenia when injected into rabbits. The two following experiments upon dogs show how marked this effect may be. In both cases the animals were anesthetized with morphine and urethane and then, after suitable controls had been obtained, received large intravenous hirudin injections. The two experiments are presented in the form of charts. In the case of the animal described through figure 8 (dog R. R.), the capillary blood just prior to hirudin injection (75 mgm. in 22 cc. of Ringer's solution) contained 123 nucleated red cells per cubic millimeter and 30,700 leucocytes. Thirty-seven minutes after the injection was completed the nucleated red cells were 100 per cubic millimeter and the leucocytes 800. Following this, there was a steady rise in leucocyte count to the end of the experiment, with a break in the rise of nucleated red cells in the neighborhood of 600 per cubic millimeter. The coagulation time rose for a while and then fell. It

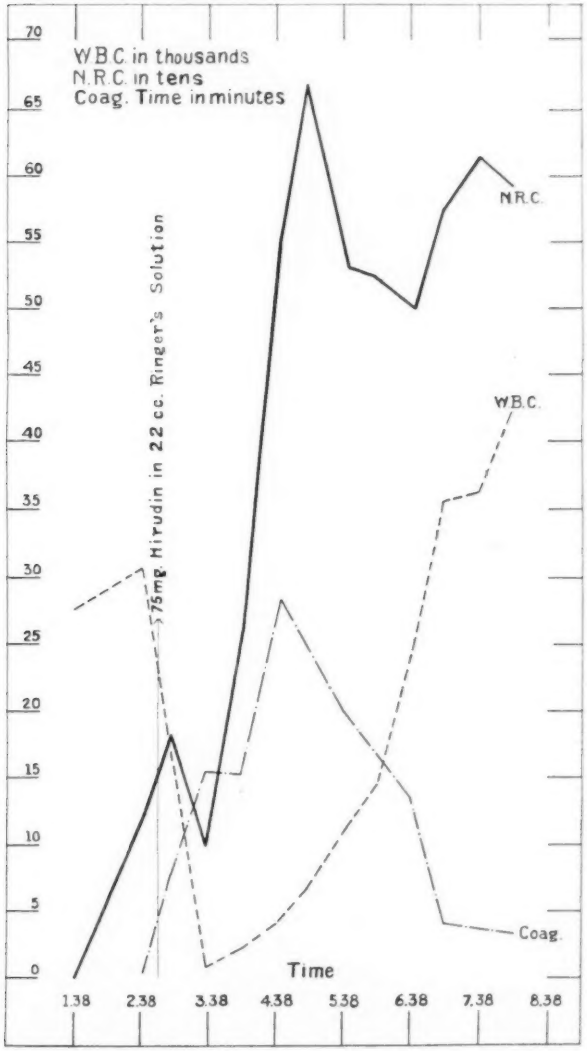

Fig. 8. Chart showing the effect of an intravenous injection of hirudin upon the leucocyte count, the nucleated red cell count and the coagulation time of dog R. R.

is of interest that in this and in other experiments the incoagulability of the blood, measured by Howell's method (19), increased for some time after the injection of the hirudin, a fact with which we were unfamiliar in regard to the action of this substance.

In the second case, figure 9, the dog (dog A) used showed 6 nucleated red corpuscles per cubic millimeter in one count made before hirudin injection, and none in another count. Ten minutes following intravenous hirudin injection (100 mgm. in 20 cc. of Ringer's solution), the leucocyte count had fallen from 16,400 per cubic millimeter to 6500 and the nucleated red cells had risen from an average of 3 per cubic millimeter to 195. As in the previous experiment, the number of these latter cells after a time reached a plateau but the leucocyte count continued to rise practically to the close of the observations.

In a third experiment a similar result was obtained and the numbers of nucleated red corpuscles found in blood, taken not only from the capillary circulation but also from the carotid artery and from the jugular vein, were found to be identical. The increases noted are, therefore, true ones and not simple matters of changed distribution.

Blood-pressure tracings taken during intravenous injection of our hirudin resulted in a slight fall of arterial pressure, with some quickening of heart beat, and then a slow return to normal relations.

These experiments make it obvious that if one perfuses a bone with blood containing hirudin one may expect this substance alone to cause dislocation of cells from the marrow. Even under the best of circumstances, the experiment does not mimic normal conditions. In spite of this, it has seemed to us that our perfusions have disclosed certain important facts. If, as in experiment U. U., the rate of flow of hirudinized blood through the marrow is increased many times without causing more than a slight or negligible increase in the number of nucleated red cells in the circulating blood, it seems clear that some agency must effectively guard the immature red cells against being washed out of the red cell forming tissue. These cells cannot be in the path of a blood stream which wanders aimlessly through a loose reticulum. Under such circumstances, an experiment of this type would cause them to be washed out in great numbers. In early experiments in which bones were perfused with oxygenated salt solutions, it was found that after a time the marrow simply washed away, making the effluent turbid, like thin pus, and giving beautiful film preparations of all the marrow-cells. This does not happen in non-hyperplastic bone-marrow when blood is used for the perfusing fluid, no matter how violent the mechani-

cal stress. In hyperplastic bone-marrow, commercial hirudin apparently acts in much the same way as anesthesia, causing disintegration of the marrow tissue along the path of the blood current.

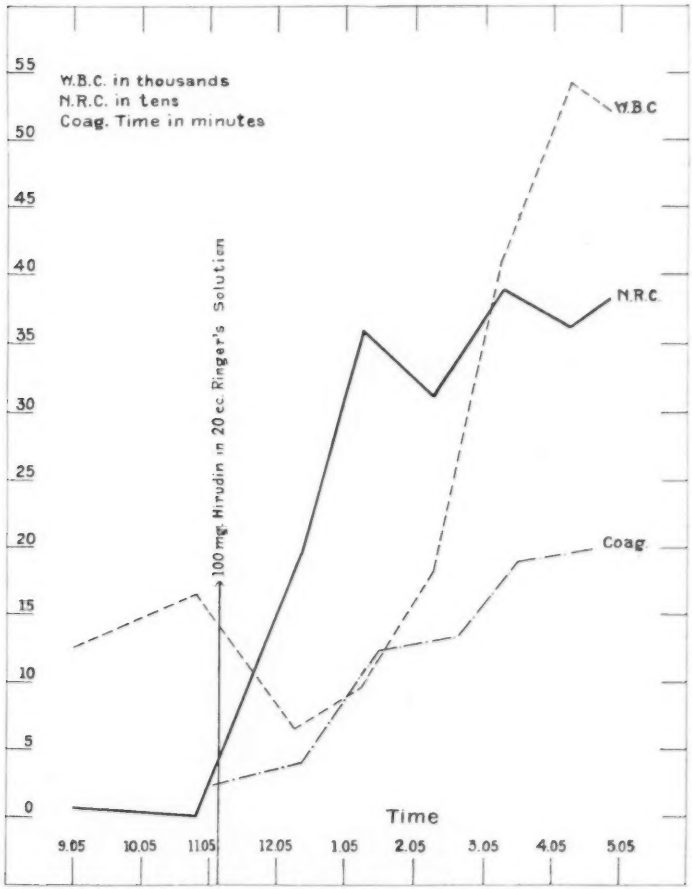

Fig. 9. Chart showing the effect of an intravenous injection of hirudin upon the leucocyte count, the nucleated red cell count and the coagulation time of dog A.

III. Perfusion experiments: *1. Methods of recording.* It is the usual belief that leucocytes leave the marrow tissue and get into the blood stream by virtue of their ameboid activity, the process being in the end quite similar to that observed when leucocytes leave capillaries and pass to a focus of irritation in the tissues, except that the line of travel in this case is reversed. The ordinary leucocyte count serves to indicate degree and rate of movement of these cells into the circulating blood. In the case of the red cells, their large numbers per cubic millimeter and their similarity make it impossible to determine movement of adult forms into the circulation unless such movement is extraordinarily pronounced. As a consequence, investigators in the field of blood formation are compelled to study the appearance in the blood stream of distinctive young forms, and, upon the basis of such appearances, to reason upon the degree of marrow activity and the manner of cellular extrusion from the marrow. In our perfusions attention was focussed upon the nucleated red cells. These cells are unmistakable in stained films and can be enumerated with a greater degree of accuracy than is possible in the case of either reticulated or polychromatophilic cells.

Throughout the experiments standardized pipettes and counting chambers were used for the red and white cell counts. Blood films made at the same time were stained with Wright's stain and the number of nucleated red cells seen during a count of at least 1000 white cells was recorded. In making a count of nucleated red cells, the following routine procedure was adopted: 500 white cells were counted in each of the two films made at every observation, and the number of nucleated red cells seen during each count recorded. If the two figures thus obtained checked satisfactorily, no more cells were counted, but if the figures were unduly divergent, a second thousand white cells was counted (two 500 white-cell counts)—occasionally a third thousand—and an average taken of all the figures obtained in this way. The average figure representing the number of nucleated red cells seen per thousand white cells was then multiplied by the number of thousand white cells per cubic millimeter present. This final figure was recorded as the number of nucleated red cells present per cubic millimeter.

In all observations cover-slip films were used and every care was taken to keep them of uniformly high quality. Even under such circumstances it is obvious that only large changes in count are of value. Our experiments indicate that such changes can be made to occur in the isolated marrow perfusions and, furthermore, that the cells of

one series, either the leucocytic or erythrocytic group, can be made to move without substantial effect upon the other group.

2. Experiments. A. Perfusion under greatly increased pressure and rate of blood flow.

Protocol 7. May 30, 1918. Dog U. U., young animal; weight, 13.2 kgm.

8:45 a.m. 60 mgm. morphine sulphate subcutaneously.

9:15 a.m. 16.5 grams urethane by stomach tube.

9:30 a.m. Specimen 1, capillary blood: leucocytes 16,800; erythrocytes 8,025,000; nucleated red cells 0.

1:10 p.m. At this time isolation of the tibia, as described in the section upon technique, was completed, cannulas were in position, and all was in readiness to bleed the animal and to fill the perfusion pump. Figure 10, tracing 1, indicates pressure relations in the artery of the left leg corresponding to the pressure in the artery containing the inflow cannula for the perfusion upon the right side. This tracing is not comparable with those made later since the lever of the membrane manometer was badly adjusted. After and including tracing 2, all records are directly comparable.

Fig. 10. Blood-pressure relations during the perfusion experiment of May 30, 1918, dog U. U.

1:13 p.m. Rate of blood flow through tibial circulation, 14.8 cc. per minute. Specimen 2, venous blood from tibia: leucocytes 33,200; erythrocytes 7,841,000; nucleated red cells 0.

1:18 p.m. Figure 10, tracing 2, pressure relations in artery of right leg similar to tracing 1. Rate of blood flow through tibial circulation, 15.2 cc. per minute. Specimen 3, venous blood from tibia: leucocytes 30,000; nucleated red cells 0.

1:28 p.m. Bled 200 cc. from left carotid artery into approximately 10 cc. of physiological salt solution containing 75 mgm. of hirudin. Pump filled with this blood and final arrangements made to start perfusion.

1:37 p.m. Specimen 4, blood taken from pump prior to passage through tibia: leucocytes 19,100; erythrocytes 7,434,000; nucleated red cells 19.

1:59 p.m. Started perfusion under pressure conditions illustrated in figure 10, tracing 3, the pressure relations obviously imitating very closely those obtaining in the control tracing 2. Rate of blood flow through the perfused tibia under these pressure relations, 9.1 cc. per minute.

2:02 p.m. Figure 10, tracing 4, indicates blood-pressure from this time until 2:16.

2:15 p.m. Specimen 5, blood taken from pump after 16 minutes tibial circulation: leucocytes 18,400; nucleated red cells 0.

2:16 p.m. Figure 10, tracing 5, shows the effect of adjusting the pump so as to give increased pressure and rate of flow.

2:21 p.m. Rate of blood flow through tibia under new pressure relations, 60 cc. per minute.

2:23 p.m. Figure 10, tracing 6, shows still further adjustment of pump.

2:29 p.m. Rate of blood flow through tibia under pressure relations shown in tracing 6, 50 cc. per minute.

2:30 p.m. Specimen 6, blood taken from the pump after 31 minutes perfusion: leucocytes 19,000; nucleated red cells 0.

2:45 p.m. Specimen 7, blood taken from the pump after 46 minutes perfusion: leucocytes 12,300; nucleated red cells 25.

2:47 p.m. Figure 10, tracing 7, shows the result of slight manipulation to increase blood-pressure again.

3:00 p.m. Specimen 8, blood taken from the pump after 61 minutes perfusion: leucocytes 13,800; nucleated red cells 0.

3:02 p.m. Figure 10, tracing 8.

3:15 p.m. Specimen 9, blood taken from the pump after 76 minutes perfusion: leucocytes 12,800; nucleated red cells 0.

3:16 p.m. Rate of blood flow through the tibia, 50 cc. per minute.

3:30 p.m. Specimen 10, blood taken from the pump after 91 minutes perfusion: leucocytes 15,200; nucleated red cells 46.

3:45 p.m. Specimen 11, blood taken from the pump after 106 minutes perfusion: leucocytes 18,200; erythrocytes 7,322,000; nucleated red cells 36.

3:57 p.m. Rate of blood flow through the tibia, 42.8 cc. per minute.

4:00 p.m. Specimen 12, blood from the pump after 121 minutes perfusion: leucocytes 17,000; nucleated red cells 17.

4:02 p.m. Shifted to injection of India ink, pressure relations remaining as in figure 10, tracing 8. Weight of bone on removal, 70 grams. Grossly very thoroughly injected. Specimens of shaft marrow taken for histological study.

Comment. In this case a normal young animal showing no nucleated red cells in his capillary blood was selected and the tibial marrow subjected to a vast increase in rate and pressure of circulation. Thus, the mean pressure recorded in figure 10, tracing 2, was 145 mm. of mercury, and a very similar pressure was maintained during the first 24 minutes of perfusion; from that time on, the pressure was increased so that during the last hour of perfusion the high figure of 240 mm. of mercury was held steadily. It will be noted that two measurements of rate of flow under the animal's own tibial circulation resulted in the figures of 14.8 and 15.2 cc. per minute. On shifting to the perfusion with hirudinized blood under practically identical pressure relations, we obtained a rate of flow of 9.1 cc. per minute. The variation is not surprising if one keeps in mind the fact that between this measurement and the two preceding the animal had been subjected to a large hemorrhage, 200 cc. of blood, to use for the perfusion. This hemorrhage may have caused a peripheral constriction, and it is possible that unavoidable variations in temperature may also have resulted in changing the caliber

of the tibial vessels. As has been explained, the arrangement of the apparatus is such that one can adjust the pump to give practically any pressure relations that may be desired, but when part of the regulating apparatus consists of a competent by-pass it is evident that very slight alterations in the caliber of the tibial vessels may be reflected in substantial variations in blood flow.

It will also be observed that after the perfusion pressure was increased a rate of flow through the tibial circulation of 60 cc. per minute was reached. This occurred at 2:21, 22 minutes after the initiation of perfusion. At 2:29 the rate was 50 cc. per minute, and the same rate was recorded at 3.16. At 3:57, just at the close of the experiment, the rate was 42.8 cc. per minute, even though the pressure had been increased. These variations are great and arise, in part, from the fact that in this experiment rates of flow were measured by recording the time taken for blood flowing from the tibia to rise 5 cc. in the graduated intake reservoir. This time was measured by a stop-watch and the method, while fairly accurate for slow rates of flow, was inadequate for the rapid flow obtained in this experiment. Thus, 5 cc. in 5 seconds means 60 cc. per minute, and 5 cc. in 6 seconds 50 cc. per minute. In spite of errors, however, it is obvious that the rate of blood flow through this bone was vastly and suddenly increased. Toward the end of the experiment the rate of flow fell off somewhat, a finding which often occurred in our earlier perfusions and which was probably due to the fact that an atmosphere of oxygen alone was used in our artificial lung. This resulted in freeing the blood of carbon dioxide, with a resulting gradual shift toward alkalinity and a consequent constriction of the smaller arterioles in the marrow. Perfusions in which blood is used should not result in edema, and we are inclined to believe that reductions in blood flow were not due to extravascular fluid.

Figure 11 is a chart of this entire perfusion experiment insofar as the blood counts and rates of flow are concerned. Throughout the experiment the nucleated red cell count varied between 0 and 46 per cubic millimeter. At 1:37, in the blood taken from the carotid artery and used for perfusion, 1 nucleated red cell was found during a count of 1000 leucocytes. This resulted in the total of 19 per cubic millimeter in this specimen. After perfusion had been in operation, no nucleated red cells were found at 2:15, 2:30, 3:00 and 3:15. Small numbers appeared in other specimens but at no time was there a pronounced outflow of these young cells. The highest figure charted (at 3:30) indicates the finding of 3 nucleated red cells during a count of 1000 leucocytes.

In this and in other experiments it will be noted that the leucocyte count in the blood used for perfusion is much lower than in blood taken from a vein a few minutes before. This is not due to dilution by salt

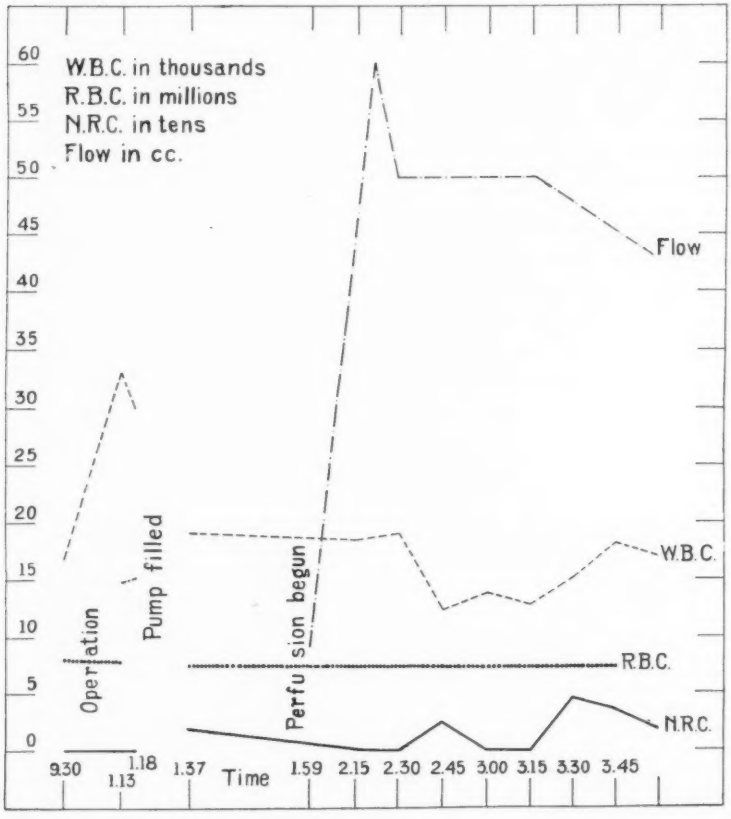

Fig. 11. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of blood-flow in the perfusion experiment of May 30, 1918, dog U. U.

solution containing hirudin, and it is not an expression of some large inequality in the leucocyte count of circulating arterial and venous blood. Bloods from these two sources will be found to be fairly uni-

form in leucocyte count if small specimens are taken, but if one opens an artery widely and allows a considerable fraction of the animal's blood to flow into an anticoagulant and then counts the leucocytes in the blood removed from the body, one will invariably find the count lower than in small blood specimens taken just previously. It seems probable that the opening of a large artery, with a very abrupt fall in blood-pressure, causes a sudden and extensive degree of arteriolar and possibly capillary constriction, thereby temporarily arresting leucocytes. Later on, these cells appear in the circulation again and thus assist in the development of the post-hemorrhagic leucocytosis, though simple failure to lose leucocytes is not the only cause for this phenomenon. The observation that the leucocyte count is normally lower in large amounts of arterial blood than in small specimens of blood taken a moment before, is well shown in table 1, which is compiled from a number of experiments.

TABLE 1

Leucocyte counts in small amounts of venous blood as contrasted with leucocyte counts in blood removed from the carotid and received into 20 cc. or less of salt solution containing hirudin

DOG	W. B. C. PER C. MM. VENOUS BLOOD	BLOOD REMOVED FROM CAROTID ARTERY IN CC.	W. B. C. PER C. MM. IN BLOOD REMOVED
U. U.	30,000	200	19,100
K. K.	30,100	400	16,800
N. N.	28,600	250	25,200
H. H.	30,900	350	10,300
M. M.	25,600	240	15,500
P. P.	23,400	200	14,700
O. O.	19,400	170	10,500
L. L.	23,000	440	10,400
Q. Q.	22,800	200	13,200
T. T.	32,100	200	23,600

The leucocytic curve in figure 11 shows a fall in count beginning about a half-hour after perfusion—a fall which is followed by a gradual rise. This is a frequent finding in the perfusions of the marrow, but in this case it has occurred somewhat slowly. Intravenous injections of foreign protein into intact animals usually cause a leucopenia followed by a leucocytosis. Figures 8 and 9, showing the effects of hirudin, are cases in point, and in such experiments there is a certain amount of evidence that circulating leucocytes are arrested in the bone-marrow, although the liver and lungs are much more definite and important

sites of arrest. It is natural, in view of the general knowledge upon leucopenia, to expect a certain degree of leucopenia when blood from an animal is mixed with a foreign protein—in this case, hirudin—and under physiological conditions passed through a tissue capable of taking part in the leucopenic reaction. The question will be asked as to whether this change is not due to adhesion of leucocytes to the tubing in the apparatus. The presence of the by-pass insures so active a movement of blood that there is no opportunity for sedimentation nor for adhesion of leucocytes to tubing. Neither does it seem to us that leucocytes break down as a result of mechanical injury by the pump and valves. Rous and Turner (20) have shown that red cells suspended in plasma are thoroughly protected against injury by shaking in glass. They do not mention the effect of shaking upon the leucocytes. In

TABLE 2

Leucocyte and nucleated red cell counts in hirudinized dog's blood subjected to rapid circulation through pump for 2 hours

TIME	LEUCOCYTES PER C. MM.	NUCLEATED RED CELLS PER C. MM.
11:40	7,200	29
12:10	7,500	8
12:25	7,000	28
12:40	6,800	41
1:10	6,800	20
1:40	6,500	20

an ordinary bone-marrow perfusion lasting an hour and a half, a slight degree of hemolysis occurs, the plasma assuming a definite pink tinge. This destruction is never visible in the red-cell count nor has it been possible, in control observations when hirudinized blood has been circulated rapidly through the pump alone for from an hour to 3 hours' time, to bring about any measurable destruction of leucocytes or of nucleated red cells. Table 2, for example, illustrates the relative constancy of the leucocyte and the nucleated red cell counts in hirudinized dog's blood which was subjected to rapid circulation through the pump for a period of 2 hours. The amount of tubing, the artificial lung, etc., were similar to those used in a marrow perfusion.

The fall and rise in leucocyte count observed in the perfusion experiment under discussion seems, then, to be the expression of the leucopenic reaction in the entire animal narrowed down to the bone-marrow alone.

At the end of the experiment the perfused tibia of dog U. U. was removed and cleaned. The weight of the tibia plus that of the fibula, which was left in place and included, was 70 grams. The tibia was then split open, and grossly seemed fully injected with the India ink introduced at the termination of the experiment. From the middle of the dog's tibia one can usually obtain a cylinder of marrow, 1 to 1½ inches in length, for microscopical sections. Marrow from this region in the dog ordinarily contains comparatively few blood-forming cells, and the cells which do exist are disposed as a shell just inside the endosteum. It is, however, impossible to obtain good histological sections of the marrow from the heads of the bone or from the shaft near the extremities owing to the spicules of bone present in such material. Decalcified specimens have proved of no value for microscopical study of the finer details of the marrow. Figure 12 is a low-power camera lucida drawing of a specimen of the injected marrow from dog U. U., and figure 13 is a high-power drawing from another part of the same marrow. Under low power it is apparent that the amount of cell-bearing marrow in the shaft of the bone is large. The irregular right border of the drawing is the endosteal side, and frequently, even in animals that have been bled, the blood cell-bearing marrow exists as an extremely thin cylinder immediately against the bone. One large vessel running through this tissue is comparatively well filled with the injection mass and it is clear that general extravasation of the ink through the tissue has not taken place. Under high power (fig. 13), two characteristic capillaries are shown and their definite lining is well made out. The stroma contains a fair number of nucleated red cells, not so many as are found in other animals in this series, but about the usual number present in a normal animal. They are evidently outside the blood current and out of reach of the vigorous flow which characterized this perfusion. The experiment shows the complete integrity of the normal marrow capillaries engaged in carrying a current of blood. Such vessels are endothelial-lined structures of simple type and seem to possess none of the peculiarities assigned to them by other investigators.

B. Perfusion under increased pressure and rate of blood flow: Typhoid vaccine added to perfusing blood.

Protocol 8. February 16, 1918. Dog N. N.; weight, 19.9 kgm.

8:45 a.m. 75 mgm. morphine sulphate subcutaneously. Specimen 1, capillary blood: leucocytes 18,200; erythrocytes 7,726,000; nucleated red cells 0.

10:25 a.m. 24.9 grams urethane by stomach tube.

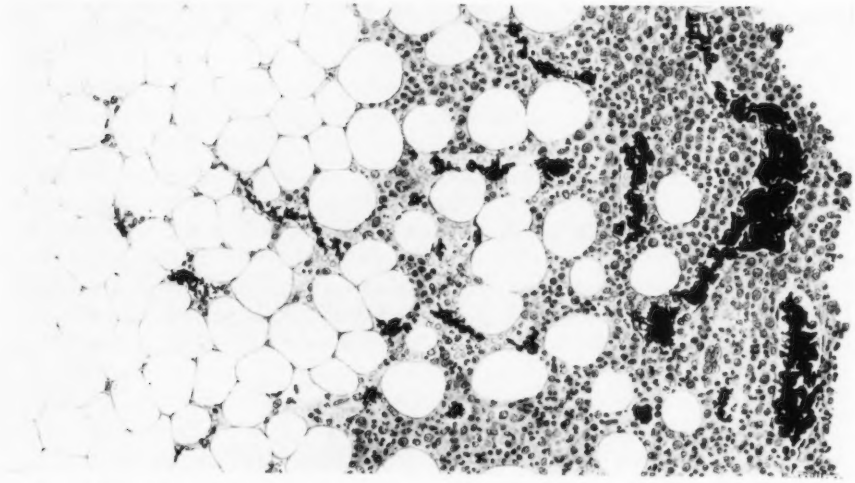

Fig. 12. Camera lucida drawing of the marrow injected at the close of the perfusion experiment of May 30, 1918, dog U. U. × 180.

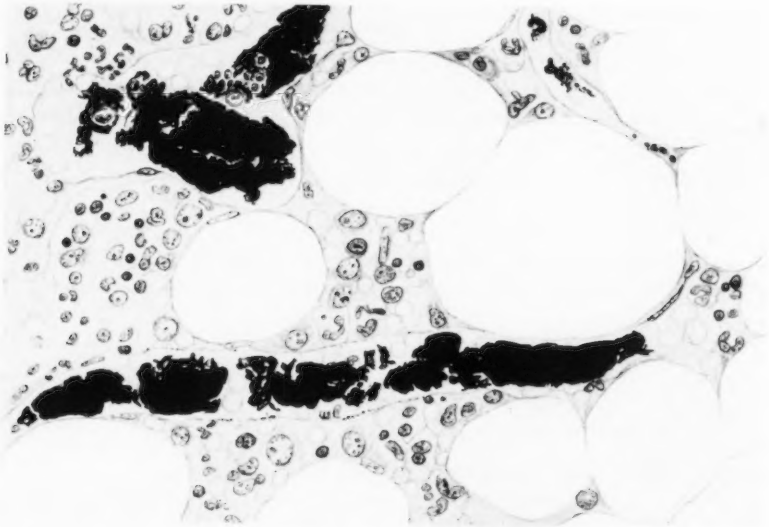

Fig. 13. Camera lucida drawing of the marrow injected at the close of the perfusion experiment of May 30, 1918, dog U. U. × 500.

10:50 a.m. Dissection for isolation of the tibia started and carried through as described in section upon technique.

12:45 p.m. Specimen 2, capillary blood: leucocytes 26,700; nucleated red cells 0.

3:10 p.m. Cannulas in position and all in readiness for perfusion. Figure 14, tracing 1, gives the pressure relations in the artery of the left leg corresponding to the pressure in the artery containing the inflow cannula for the perfusion on the right side.

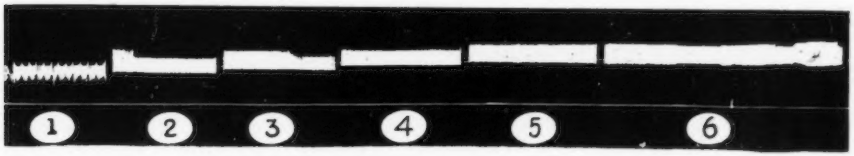

Fig. 14. Blood-pressure relations during the perfusion experiment of February 16, 1918, dog. N. N.

3:15 p.m. Rate of the blood flow through tibial circulation, 28.8 cc. per minute. Specimen 3, venous blood from tibia: leucocytes 28,600; erythrocytes 7,385,000; nucleated red cells 29.

3:21 p.m. Rate of blood flow through tibial circulation, 28.2 cc. per minute. Specimen 4, venous blood from tibia: nucleated red cells 0.

3:23 p.m. Bled 250 cc. into approximately 10 cc. of physiological salt solution containing 75 mgm. of hirudin. Pump filled with this blood and final adjustments made to start perfusion.

3:25 p.m. Specimen 5, blood taken from the pump prior to passage through tibia: leucocytes 25,200; erythrocytes 7,097,000; nucleated red cells 0.

3:37 p.m. Perfusion started under pressure relations shown in figure 14, tracing 2. The mean pressure in the control tracing 1 is 140 mm. of mercury; that in tracing 2, 160 mm. of mercury.

3:45 p.m. Rate of blood flow through the perfused tibial vessels, 46 cc. per minute.

3:50 p.m. Specimen 6, blood taken from the pump after 13 minutes tibial circulation: leucocytes 23,000; erythrocytes 7,065,000; nucleated red cells 0.

3:53 p.m. Rate of blood flow through the perfused tibial vessels, 42 cc. per minute.

3:54 p.m. 0.5 cc. commercial typhoid vaccine (500,000,000 organisms) in 5 cc. physiological sodium chloride solution placed in pump blood.

4:00 p.m. Rate of blood flow through perfused tibial vessels, 43 cc. per minute.

4:15 p.m. Specimen 7, blood taken from the pump after 38 minutes tibial circulation: leucocytes 17,100; nucleated red cells 0.

4:16 p.m. Figure 14, tracing 3, shows the perfusion pressure at this time—168 mm. of mercury.

4:17 p.m. Rate of blood flow through the perfused tibial vessels under these pressure relations, 42 cc. per minute.

4:40 p.m. Specimen 8, blood taken from the pump after 63 minutes tibial circulation: leucocytes 19,600; nucleated red cells 0.

4:46 p.m. Rate of blood flow through perfused tibial vessels, 45 cc. per minute.

4:50 p.m. Figure 14, tracing 4, shows a further slight increase in perfusion pressure—185 mm. of mercury.

5:05 p.m. Specimen 9, blood taken from the pump after 88 minutes tibial circulation: leucocytes 18,600; nucleated red cells 19.

5:22 p.m. Rate of blood flow through the perfused tibial vessels under the pressure relations shown in figure 14, tracing 4, 40 cc. per minute.

5:30 p.m. Specimen 10, blood taken from the pump after 113 minutes tibial perfusion: leucocytes 16,000; nucleated red cells 0.

5:40 p.m. Rate of blood flow through the perfused tibia under pressure relations shown in figure 14, tracing 5, the mean pressure having been increased to 198 mm. of mercury, 36 cc. per minute.

5:50 p.m. Rate of blood flow under similar conditions, 33 cc. per minute.

5:54 p.m. Specimen 11, blood taken from the pump after 137 minutes tibial perfusion: leucocytes 13,500; erythrocytes 7,184,000; nucleated red cells 40.

Figure 14, tracing 6, indicates the pressure at the close of the experiment and during India ink injection. Weight of bone on removal, 70 grams. Grossly very thoroughly injected. Specimens of shaft marrow taken for histological study.

Comment. In this experiment an animal showing no nucleated red cells in his capillary blood was subjected to tibial marrow perfusion for 137 minutes. During this time the mean blood-pressure was always higher than that presented by the animal, starting at 160 mm. of mercury as against 140 mm. of mercury in the case of the control and rising slowly to about 200 mm. of mercury at the close of the perfusion. The blood flow, shown in figure 15, under the animal's own circulation before perfusion was instituted, on two estimations gave 28.8 and 28.2 cc. per minute. On starting perfusion under somewhat increased blood-pressure, the rate of flow was 46 cc. per minute and did not fall below 40 cc. until the last half-hour of the experiment, when it slowly fell to 33 cc. per minute. The experiment is quite comparable to that which just precedes it except that such extreme conditions of flow increase were not imposed.

Just as in the previous experiment, there was no tendency for nucleated red corpuscles to leave the marrow.

The leucocyte count fell between the beginning and the end of the experiment. The introduction of 0.5 cc. of typhoid vaccine at 3:54 was followed by a slight fall in leucocyte count, then a fairly steady level until 5:05 when a drop began which continued to the end of the experiment. The typhoid vaccine, being another foreign protein, apparently merely added to the effect of the hirudin.

Microscopical preparations of the marrow show fewer nucleated red cells than in the case of dog U. U., whose injected marrow is shown in figures 12 and 13. Marrow films show nucleated red cells in moderate numbers and, at the time of injection, the tissue was apparently in the normal formative equilibrium for the dog. The marrow capillaries

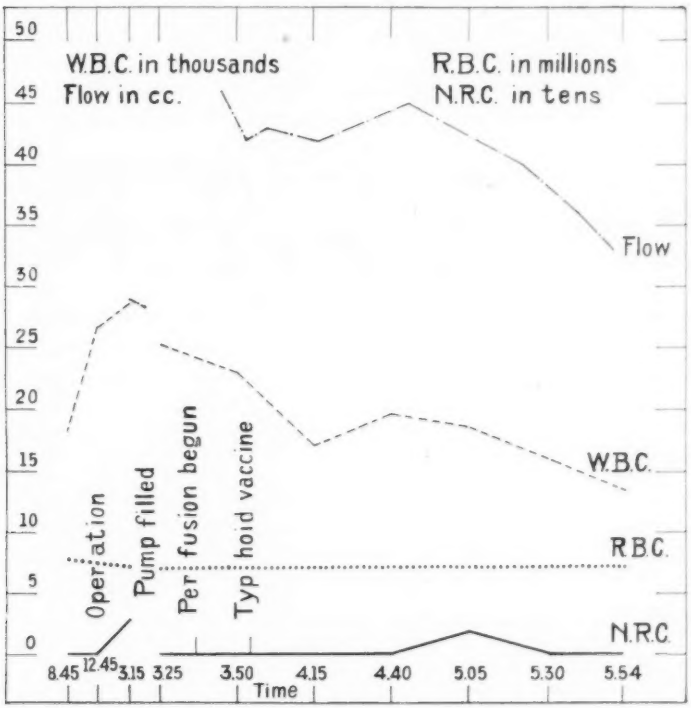

Fig. 15. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of blood flow in the perfusion experiment of February 16, 1918, dog N. N.

show good filling with the ink injection, and, in an unusually large number of cases, a definite complete endothelial lining can be made out. This is of interest since, as will be seen later, irregularity and indefiniteness of capillary outline are frequent and valuable indications of active marrow growth. There is no evidence of capillary rupture

with diffusion of ink through the marrow substance, nor are there recognizable intracapillary collections of leucocytes. The leucopenia which appears most significantly toward the end of the experiment is accompanied by a fall in blood-flow through the marrow. It had been our expectation that this might be shown to be due to blockage by groups of leucocytes within the capillaries, since it has been observed that in the stage of leucopenia following intravenous injections of foreign protein into intact animals there is a noticeable intracapillary accumulation of leucocytes in the liver, in the lungs, in the spleen, and possibly in the bone-marrow, though in this latter case difficulties in capillary definition have made it doubtful as to whether the leucocytes in question had not migrated into the marrow substance. The fact that in most instances the capillaries were well filled with ink in this experiment makes it possible to look for leucocyte accumulations only in cases in which the injection was not quite complete, a feature of the situation which again makes histological explanation of the leucopenia impossible.

The experiment may be summarized as adding confirmation to the evidence presented in the previous more extreme case—namely, that mechanical measures alone will not disturb the integrity of the normal bone-marrow vasculature.

C. Perfusion of hyperplastic marrow under increased pressure and rate of blood flow.

Protocol 9. March 22, 1922. Dog D. D. D., young female; weight, 7.2 kgm. This animal was a partly grown healthy dog selected for the experiment because she was in the midst of a period of exceedingly active extrusion of young blood-cells.

12:40 p.m. Specimen 1, capillary blood: leucocytes 19,800; erythrocytes 7,244,000; nucleated red cells 5474.

12:55 p.m. 18 grams urethane by stomach tube.

1:20 p.m. Vomited.

1:35 p.m. 5 grams urethane by stomach tube.

5:21 p.m. Dissection finished and preparations for perfusion completed. These preparations included the removal of 200 cc. of blood from another dog. This blood was received into 28 cc. of Ringer's solution containing 225 mgm. of hirudin. The large amount of hirudin employed was necessitated by the inefficacy of the post-war preparations which were available. Owing to the extraordinary number of normoblasts in the circulation of dog D. D. D. at the time of the experiment, it was thought wise to use blood from another animal. Figure 16, tracing 1, indicates the pressure relations in the artery of the left leg corresponding to the vessel in the right leg containing the inflow cannula for the perfusion. The mean pressure was 116 mm. of mercury.

5:28 p.m. Specimen 2, venous blood from tibia: leucocytes 25,800; erythrocytes 8,841,000; nucleated red cells 8204.

5:33 p.m. Rate of blood flow through tibial circulation, 9.2 cc. per minute.

5:36 p.m. Rate of blood flow through tibial circulation, 8.4 cc. per minute.

6:02 p.m. Specimen 3, blood taken from pump prior to passage through tibia: leucocytes 5200; erythrocytes 5,280,000; nucleated red cells 21.

6:03 p.m. Perfusion begun under pressure conditions shown in figure 16, tracing 2. Mean pressure, 128 mm. of mercury.

6:06 p.m. Rate of blood flow through perfused tibia, 30.8 cc. per minute.

6:14 p.m. Figure 16, tracing 3, pressure relations at this time. Rate of blood flow through perfused tibia, 30.8 cc. per minute.

6:22 p.m. Specimen 4, blood taken from the pump after 19 minutes passage through tibia: leucocytes 8500; nucleated red cells 1165.

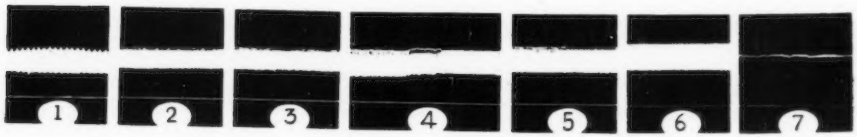

Fig. 16. Blood-pressure relations during the perfusion experiment of March 22, 1922, dog D. D. D.

6:23 p.m. Rate of blood flow through perfused tibia, 26 cc. per minute.

6:24 p.m. Figure 16, tracing 4, pressure relations at this time.

6:25 p.m. Rate of blood flow through perfused tibia, 30 cc. per minute.

6:29 p.m. Rate of blood flow through perfused tibia, 29 cc. per minute.

6:34 p.m. Rate of blood flow through perfused tibia, 26 cc. per minute.

6:37 p.m. Rate of blood flow through perfused tibia, 26 cc. per minute.

6:40 p.m. Blood beginning to ooze slowly from the region of the thigh section on the perfused side.

6:42 p.m. Figure 16, tracing 5, indicates pressure relations at this time. Mean pressure, 134 mm. of mercury.

Specimen 5, blood taken from the pump after 39 minutes passage through tibia: leucocytes 14,900; nucleated red cells 2935.

6:47 p.m. Rate of blood flow through perfused tibia, 24 cc. per minute.

6:55 p.m. Rate of blood flow through perfused tibia, 22 cc. per minute.

7:00 p.m. Rate of blood flow through perfused tibia, 20 cc. per minute.

7:01 p.m. Figure 16, tracing 6, indicates pressure relations at this time. Mean pressure, 140 mm. of mercury.

7:02 p.m. Specimen 6, blood taken from the pump after 59 minutes passage through tibia: leucocytes 10,700; nucleated red cells 3221.

7:05 p.m. Rate of blood flow through perfused tibia, 20 cc. per minute.

7:07 p.m. Rate of blood flow through perfused tibia, 18 cc. per minute.

7:12 p.m. Rate of blood flow through perfused tibia, 16 cc. per minute.

7:15 p.m. Specimen 7, blood taken from the pump after 72 minutes passage through tibia: leucocytes 15,400; erythrocytes 5,611,000; nucleated red cells 5184.

At this time it was necessary to cease perfusion since the slight constant loss of blood, first noted at 6:40, had resulted in exhaustion of the blood in the perfusion system and in the introduction of air into the pump. None of the air drawn in reached the perfused bone, but, owing to the introduction into the system of this extra elasticity, pulse pressure fell at once and was extremely low during tracing 7, figure 16, which represents conditions during the period of bone injection. The tibia, on removal, weighed 36 grams and on section showed grossly a thorough distribution of the injection mass.

Comment. Figure 17 is a chart which summarizes this entire experiment. Note, in the first place, the extraordinary number of nucleated red cells per cubic millimeter in the peripheral blood of this dog before perfusion. As a result of the anesthesia and operation, there was an increase in the number of these cells from 5474 to 8204. The blood used for the perfusion contained 21 nucleated red cells per cubic millimeter. After 19 minutes passage through the bone, the number per cubic millimeter was 1165. Some of the cells making up this first increase were probably intravascular at the time the perfusion began but enrichment from this source cannot have been great since, as in all perfusions, the first blood issuing from the venous return on institution of the artificial circulation was allowed to run out on the table, in order to avoid the possibility of clotting in the venous reservoir which might readily arise through mixture of this coagulable blood with the first hirudinized blood. The rate of blood flow during the first 20 minutes of perfusion was high, averaging 29.2 cc. per minute. At this rate, all the blood in the perfusion system had opportunity to pass through the tibia at least twice during these 20 minutes. As a consequence, it seems reasonable to consider that the degree of enrichment of the perfusing blood with nucleated red cells, intravascular at the moment of starting the perfusion, must certainly have been completed during this first period. The cells, however, continued to increase during the remainder of the experiment, reaching 5184 per cubic millimeter in the last specimen taken. The leucocyte count rose also but not to such a considerable degree.

Blood flow through the bone during perfusion was always above the figure obtained for the circulation prior to perfusion. The difference is greater than one would expect in the face of pressure conditions only slightly above those of the animal, and is probably ascribable to the lower cell content of the blood used for perfusion. The fall in the rate of blood flow is to some extent apparent, rather than real. At 6:40 it was noticed that blood was leaking slowly from the perfused part, coming from small veins in the cut muscles of the thigh. This

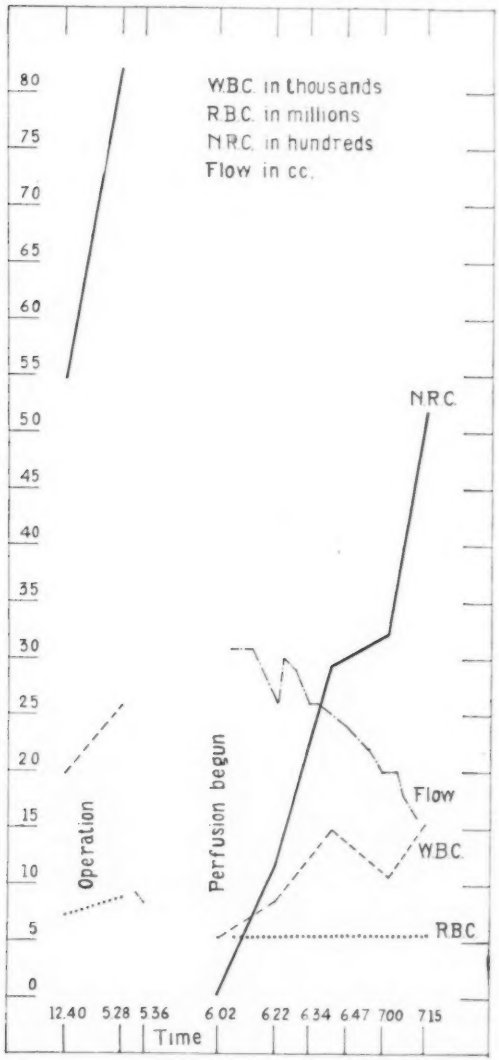

Fig. 17. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of flow in the perfusion experiment of March 22, 1922, dog D. D. D. Note that in this case the nucleated red cells are charted in hundreds per cubic millimeter and not in tens as in other charts throughout the paper.

loss eventually terminated the experiment, and, during the course of the experiment, constantly reduced the blood collected into the perfusion system. As a consequence, after 6:40 and probably prior to this time, readings upon the rate of blood flow were undoubtedly low and did not represent the blood actually passing through the tibia.

On both gross and microscopical examination, the marrow of this tibia proved intensely hyperplastic. Its condition is well displayed in figure 18. The upper drawing shows four capillaries, no one of which displays any trace of endothelial lining. In all cases the capillary outline is irregular, and in one capillary, *X*, the injected ink surrounds several nucleated red cells which are obviously in a position favorable for removal from the marrow. The capillaries in the lower two drawings show—in the one instance, *Y*—a blood space with a comparatively smooth wall, but without any evidence of endothelial lining, in close apposition to a large colony of nucleated red blood cells. The other capillary, *Z*, displays the same lack of wall, irregularity of outline and intimate relation to nucleated red cells seen in the upper drawing. While the illustrations display the typical relations of circulating blood and marrow in this animal at the moment of injection, areas can be found in which the injected ink has wandered some distance into the marrow stroma, the condition being similar to but less extensive than that seen in figures 43 and 44, which illustrate the condition of the marrow after saponin poisoning has destroyed the vascular outlines.

This experiment contrasts markedly with those which precede it. In all three cases animals in perfect health and apparently maintaining a normal number of red cells in their blood were perfused at a more rapid rate of blood flow than is normal for the tibial marrow. In the first two cases, dogs U. U. and N. N., the marrow capillaries show a distinct endothelial lining separating the marrow stroma from the blood current. Tibial perfusion in these animals resulted in no dislocation of nucleated red cells. In the last case, dog D. D. D., a puppy in the midst of a period of extremely active red cell growth, was subjected to a similar perfusion which resulted in the appearance of large numbers of nucleated red cells in the perfusing blood. These cells were leaving the marrow in large numbers at the time when perfusion was instituted and the artificial circulation merely intensified what was already in progress. In two other experiments upon hyperplastic animals perfused at periods of active cellular extrusion from the marrow, similar rapid enrichment of the perfusing blood with immature red cells was obtained. One is therefore forced to conclude that an exces-

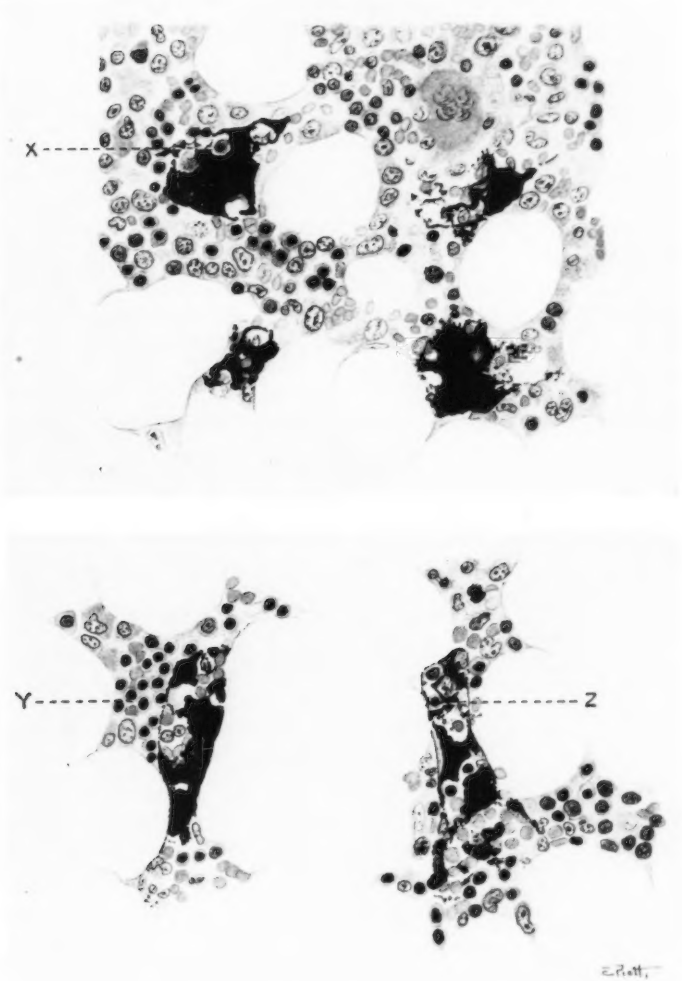

Fig. 18. Camera lucida drawing of two areas in the marrow injected at the close of the perfusion experiment of March 22, 1922, dog D. D. D. × 500.

sively rapid blood flow through bone-marrow which is extruding nucleated red cells at the moment when the rapid flow is instituted, favors the dislocation of these cells from the tissue. It is true that anesthesia, operation and the use of hirudin may play a part in producing this result, but the fact remains that it will invariably occur in bone-marrow which is losing nucleated red cells at the moment of perfusion and will not occur in marrow which is not losing nucleated red cells, even though the technical procedures involved are the same. In a later section of this work the capillary relations in hyperplastic bone-marrow, unsubjected to perfusion, are shown, and it becomes easy to understand that growth encroachment of marrow cells upon the blood vessels places immature cells in a favorable position for removal. It is not surprising, therefore, that they are unable to remain within the marrow when subjected to an excessively rapid blood current, such as was used in this experiment.

D. Perfusion under moderate pressure and slow rate of blood flow: Saturation of blood with carbon monoxide to produce complete asphyxia of the marrow early in the perfusion.

Protocol 10. Dog J. J., male; weight, 23 kgm.

In order to ensure an hyperplastic marrow this animal was bled under morphine anesthesia, prior to the perfusion, as follows:

August 20, 1917. Bled 800 cc. Infused 900 cc. of physiological salt solution during this hemorrhage.

September 6, 1917. Bled 750 cc. Infused 800 cc. of physiological salt solution during this hemorrhage.

September 19, 1917. Bled 700 cc. Infused 850 cc. of physiological salt solution during this hemorrhage.

October 13, 1917. Bled 650 cc. Infused 900 cc. during this hemorrhage.

Such methods of bleeding and infusion give no idea of the exact amount of blood removed, since some of the fluid taken out has just been introduced. The procedure, however, permits one to bleed the animal very extensively, our aim in this case.

November 3, 1917. *Perfusion.* Weight 16.1 kgm. On August 20, when first bled, this dog weighed 23 kgm. The loss of weight was not coupled with loss of vigor, and at the time of perfusion the health of the animal was apparently excellent.

8:30 a.m. 75 mgm. morphine sulphate subcutaneously.

9:15 a.m. 20.1 grams urethane by stomach tube.

9:32 a.m. Specimen 1, capillary blood: leucocytes 13,100; erythrocytes 7,802,000; nucleated red cells 0.

1:30 p.m. Isolation of the tibia completed in the manner already described, cannulas in place, and preparations for filling perfusion pump and starting perfusion completed.

2:33 p.m. Figure 19, tracing 1, indicates the pressure relations in the artery of the left leg corresponding to the vessel in the right leg containing the inflow cannula for the perfusion. Mean pressure, 120 mm. of mercury.

2:42 p.m. Rate of blood flow through tibial circulation, 2.6 cc. per minute.

Specimen 2, venous blood from tibia: leucocytes 26,200; erythrocytes 7,909,000 nucleated red cells 0.

2:47 p.m. Specimen 3, venous blood from tibia: leucocytes 28,400; erythrocytes 7,447,000; nucleated red cells 19 (1 cell seen in counting 1500 leucocytes in film).

Figure 19, tracing 2, indicates pressure relations under conditions similar to tracing 1—the dog's own circulation.

Rate of blood flow under these conditions, 2.6. cc. per minute.

2:47–2:53 p.m. The pump was now filled with 420 cc. of hirudinizd blood taken from another dog, H. H., in order to try the effect of avoiding a large hemorrhage immediately prior to perfusion. No serological tests as to the compatability of this blood with the blood of the perfused animal were made, nor was record kept of the amount of hirudin employed. Presumably between 75 and 100 mgm. were used.

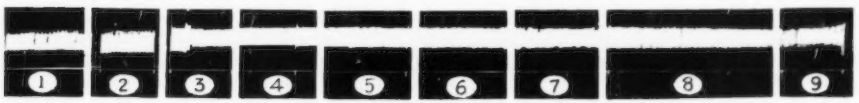

Fig. 19. Blood-pressure relations during the perfusion experiment of November 3, 1917, dog J. J.

Specimen 4, blood taken from pump prior to passage through tibia: leucocytes 17,800; erythrocytes 5,781,000; nucleated red cells 0.

Perfusion started under pressure conditions shown in figure 19, tracing 3. The mean pressure in this case was 140 mm. of mercury as against 120 mm. in the last control, figure 19, tracing 2.

2:55 p.m. Rate of blood flow through the perfused tibia under these pressure relations, 10.2 cc. per minute.

3:06 p.m. Rate of blood flow through perfused tibia, 6 cc. per minute.

3:08 p.m. Specimen 5, blood taken from the pump after 15 minutes passage through tibia: leucocytes 19,600; erythrocytes 6,846,000; nucleated red cells 26.

Figure 19, tracing 4, pressure relations at this time.

3:10 p.m. Pure carbon monoxide, made by the action of sulphuric acid on formic acid and washed through sodium hydroxide, was now introduced through one of the tubes in the top of the artificial lung, no. 7, figure 7. The first gas introduced was run out into the room and washed air out of the lung with it. For the remainder of the experiment carb on monoxide passed steadily through the lung so that the perfusing blood was not only promptly entirely saturated with carbon monoxide but by the extreme conditions imposed was kept so to the close of the experiment.

Rate of blood flow through the perfused tibia, 5.8 cc. per minute.

3:15 p.m. Rate of blood flow through the perfused tibia, 5.8 cc. per minute.

3:20 p.m. Rate of blood flow through the perfused tibia, 6.3 cc. per minute.
3:23 p.m. Specimen 6, blood taken from pump after 30 minutes tibial circulation: leucocytes 15,900; nucleated red cells 0.
3:26 p.m. Rate of blood flow through the perfused tibia, 6.2 cc. per minute.
3:28 p.m. Figure 19, tracing 5, pressure relations at this time.
3:32 p.m. Rate of blood flow through the perfused tibia, 5.8 cc. per minute.
3:37 p.m. Rate of blood flow through the perfused tibia, 6.0 cc. per minute.
3:38 p.m. Specimen 7, blood taken from pump after 45 minutes tibial circulation: leucocytes 18,100; erythrocytes 6,183,000; nucleated red cells 9 (1 nucleated red cell seen in counting 2000 leucocytes in film).
3:44 p.m. Rate of blood flow through the perfused tibia, 5.7 cc. per minute.
3:45 p.m. Figure 19, tracing 6, pressure relations at this time.
3:49 p.m. Rate of blood flow through the perfused tibia, 5.5 cc. per minute.
3:53 p.m. Specimen 8, blood taken from pump after 60 minutes tibial circulation: leucocytes 18,600; nucleated red cells 0.
3:55 p.m. Rate of blood flow through the perfused tibia, 5.3 cc. per minute.
4:00 p.m. Rate of blood flow through the perfused tibia, 5.2 cc. per minute.
4:01 p.m. Figure 19, tracing 7, pressure relations at this time.
4:05 p.m. Rate of blood flow through the perfused tibia, 5.2 cc. per minute.
4:08 p.m. Specimen 9, blood taken from pump after 75 minutes tibial circulation: leucocytes 21,300; nucleated red cells 0.
4:10 p.m. Rate of blood flow through the perfused tibia, 5.0 cc. per minute.
4:16 p.m. Rate of blood flow through the perfused tibia, 4.7 cc. per minute.
4:21 p.m. Rate of blood flow through the perfused tibia, 4.2 cc. per minute.
4:23 p.m. Rate of blood flow through the perfused tibia, 4.2 cc. per minute.
Specimen 10, blood taken from the pump after 90 minutes tibial circulation: leucocytes 20,300; erythrocytes 6,741,000; nucleated red cells 81 (8 nucleated red cells seen in counting 2000 leucocytes in the film).
4:26 p.m. Figure 19, tracing 8, pressure relations at this time.
4:33 p.m. Figure 19, tracing 9, pressure relations at the close of the injection. After cleaning, the bone weighed 77 grams. It was very thoroughly injected.

Comment. In this experiment the rate of flow was consistently low, a very brief period immediately after starting the perfusion being excepted. Measurements of the flow under the animal's own circulation prior to the beginning of the artificial circulation gave 2.6 cc. per minute on two different sets of measurements. Under the slightly higher pressure used for perfusion this was always exceeded and, though there was a fall toward the end of the experiment, the average flow during perfusion—excluding the 10.2 cc. reading obtained at the outset—was 5.4 cc. The blood used for perfusion was taken from another dog in order to avoid the necessity for hemorrhage in the animal under observation. An unusually large amount, 420 cc., was employed and in the 90 minutes of the perfusion at an average rate of flow of 5.4 cc. per minute, 486 cc. of blood actually passed through the bone. This

means that the blood studied for cellular enrichment had little more than a single passage through the marrow in which to pick up cells, and it is not surprising that no increase in nucleated red cells is noted until the last specimen, which shows what is probably the beginning of a profuse extrusion.

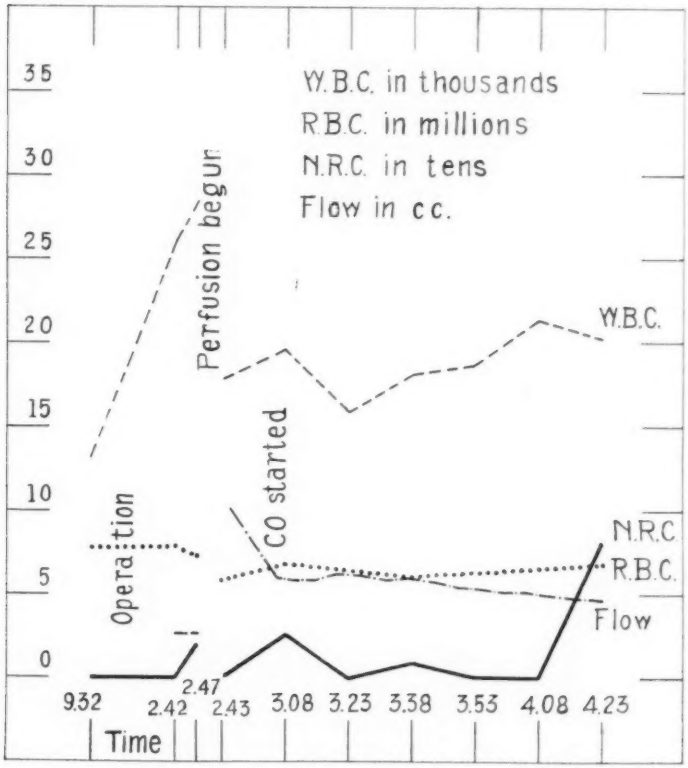

Fig. 20. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of flow in the perfusion experiment of November 3, 1917, dog J. J.

Reference to figure 20 shows that the leucocyte count after starting perfusion rises slightly. This is followed by a fall with a subsequent tendency toward leucocytosis. The saturation of the blood with pure

carbon monoxide at 3:10 seems to have produced little effect. In two subsequent experiments it will be seen that the complete asphyxia produced in this way is soon followed by a rather general outpouring of marrow cells into the circulation. In view of other findings, it seems that such an effect was about to take place in this experiment, but its termination at 4:23 occurred just before a large degree of cellular extrusion began.

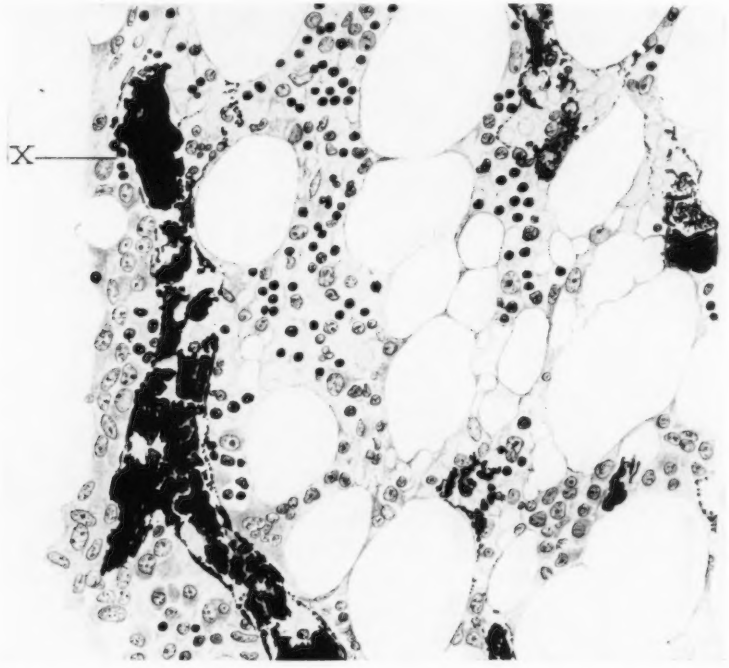

Fig. 21. Camera lucida drawing of the marrow injected at the close of the perfusion experiment of November 3, 1917, dog J. J. × 500.

On microscopical examination, as shown in figure 21, the marrow from the shaft of this tibia is seen to contain many nucleated red corpuscles and these are in their characteristic position outside the moving blood current. Capillary walls are not visible except at occasional points, but, in spite of this, there is no passage of ink through the marrow stroma. The section drawn represents the relation between blood cur-

rent and marrow as far as one can decide upon it through careful study of many specimens. It is, however, obvious that the relation is an extraordinarily delicate one, and if, as has been implied, a free delivery of leucocytes and of nucleated red cells into the blood stream is about to take place, it is clear that the first steps in the process may be of such subtlety as to elude microscopic detection. Suppose, for example, that as a result of the asphyxia following the introduction of carbon monoxide the capillary wall at *X* is broken down. Four nucleated red cells would at once be in a position to be thrown into the blood current, but in its first steps the process might not be attended by much diffusion of ink into the marrow stroma.

It has been shown (21) that after hemorrhage delivery of nucleated red cells into the blood stream occurs during a definite spurt or crisis and then stops, although the number of nucleated red cells in the marrow at the time of cessation of extrusion may be extremely high. The animal perfused in this experiment, J. J., would seem to be in such a condition. He retains the hyperplasia induced by four large hemorrhages but at the moment of the perfusion his marrow simply occupies a larger share of the medullary cavity, and does this in absence of any crowding, owing to absorption of fat and possibly even of bone. Under such circumstances, columns of nucleated red cells are not pressed out into the blood stream and, as a consequence, radically injurious measures such as the complete asphyxia produced by carbon monoxide saturation of the perfusing blood are necessary to cause cell removal.

E. Perfusion under moderate pressure and slow rate of blood flow: Saturation of blood with carbon monoxide late in the perfusion.

Protocol 11. November 24, 1917. Dog K. K., weight 17.75 kgm.

8:50 a.m. 75 mgm. morphine sulphate subcutaneously.

9:49 a.m. 22.1 grams urethane by stomach tube.

10:05 a.m. Specimen 1, capillary blood: leucocytes 28,200; erythrocytes 6,664,000; nucleated red cells 239.

1:30 p.m. Isolation of the tibia accomplished in the usual manner.

Specimen 2, capillary blood: leucocytes 31,300; erythrocytes 7,511,000; nucleated red cells 225.

Cannulas in position and all in readiness to bleed the animal and fill perfusion pump.

1:34 p.m. Figure 22, tracing 1, indicates pressure relations in the artery of the left leg corresponding to the pressure in the artery containing the inflow cannula for the perfusion upon the right side.

1:37 p.m. Specimen 3, venous blood from tibia: leucocytes 30,000; erythrocytes 7,006,000; nucleated red cells 336.

Rate of blood flow through the tibial circulation, 12.8 cc. per minute.

1:44 p.m. Figure 22, tracing 2, similar to tracing 1, shows the pressure relations in the tibia under the conditions provided by the animal.

1:50 p.m. Specimen 4, venous blood from tibia: leucocytes 30,100; erythrocytes 6,997,000; nucleated red cells 277.

Rate of blood flow through the tibial circulation, 14.8 cc. per minute.

1:54 p.m. 400 cc. of blood taken from left carotid into 75 to 100 mgm. of hirudin. Pump filled with 300 cc. of this blood. Trouble with pump caused a delay of almost one hour at this time.

2:40 p.m. Figure 22, tracing 3, indicates the reduction of pressure resulting from the removal of blood.

2:44 p.m. Specimen 5, venous blood from tibia: leucocytes 11,800; erythrocytes 6,052,000; nucleated red cells 288.

Rate of blood flow through the tibial circulation under reduced conditions of pressure, 3.6 cc. per minute.

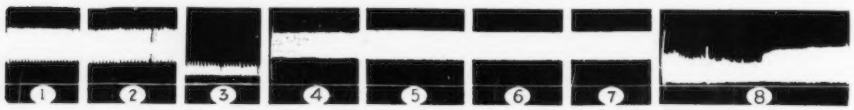

Fig. 22. Blood-pressure relations during the perfusion experiment of November 24, 1917, dog K. K.

2:46 p.m. Perfusion started under pressure shown in figure 22, tracing 4.

Specimen 6, blood taken from pump prior to passage through tibia: leucocytes 16,800; erythrocytes 5,994,000; nucleated red cells 188.

3:00 p.m. Rate of blood flow through the perfused tibia, 8.0 cc. per minute.

3:10 p.m. Rate of blood flow through the perfused tibia, 5.8 cc. per minute.

Specimen 7, blood taken from pump after 24 minutes tibial circulation: leucocytes 17,400; erythrocytes 6,135,000; nucleated red cells 219.

3:20 p.m. Rate of blood flow through the perfused tibia, 4.7 cc. per minute.

3:27 p.m. Figure 22, tracing 5, pressure relations at this time.

3:30 p.m. Rate of blood flow through the perfused tibia, 5.2 cc. per minute.

Specimen 8, blood taken from pump after 44 minutes tibial circulation: leucocytes 21,900; nucleated red cells 184.

3:40 p.m. Rate of blood flow through the perfused tibia, 4.6 cc. per minute.

3:46 p.m. Pure carbon monoxide allowed to flow through artificial lung, under conditions similar to those in preceding experiment.

3:48 p.m. Figure 22, tracing 6, pressure relations at this time.

3:50 p.m. Specimen 9, blood taken from pump after 64 minutes tibial circulation: leucocytes 22,500; nucleated red cells 225.

3:55 p.m. Rate of blood flow through the perfused tibia, 4.7 cc. per minute.

4:05 p.m. Rate of blood flow through the perfused tibia, 6.4 cc. per minute.

4:06 p.m. Figure 22, tracing 7, pressure relations at this time.

4:11 p.m. Specimen 10, blood taken from the pump after 85 minutes tibial circulation: leucocytes 21,600; nucleated red cells 315.

4:18 p.m. Valve mechanism on perfusion pump gave way, bringing about a considerable degree of regurgitation.

4:29 p.m. Specimen 11, blood taken from the pump after 103 minutes tibial perfusion: leucocytes 23,700, erythrocytes 6,394,000; nucleated red cells 384.

Figure 22, tracing 8, indicates pressure relations after 4:18 and during injection of the bone-marrow with India ink. The large pulse-pressure due to the regurgitation was at once reduced by manipulation of the pulse-pressure regulating mechanism of the pump, but a low mean pressure remained. Owing to the low pressure, the ink injection was continued for five minutes and when the bone was opened at removal injection seemed exceedingly thorough. The bone was not weighed.

Comment. Adult dogs, cared for under the ordinary conditions of laboratory life, often show nucleated red corpuscles in the circulation. In twenty-six such animals an average of 227 nucleated red corpuscles per cubic millimeter of blood has been reported, with an extreme instance in one case of 2485 nucleated red cells (22).

The dog selected for the perfusion experiment just described had 239 nucleated red cells per cubic millimeter of blood when anesthesia was complete. He was, therefore, losing young forms into the circulation at the time perfusion was instituted and the experiment was designed to test the effect, under these circumstances, of a slow rate of perfusion with blood saturated with carbon monoxide. The rate of blood flow prior to withdrawal of blood to fill the perfusion pump was 12.8 and 14.8 cc. per minute on two different determinations. It is of interest that this rate fell to 3.6 cc. per minute shortly after the hemorrhage and during the period of low blood-pressure shown in figure 22, tracing 3. The condition of affairs reflected in the low blood-pressure and low rate of blood flow lasted 52 minutes. After perfusion was started under the conditions of pressure seen in figure 22, tracing 4, the rate of blood flow, although momentarily 8 cc., remained in the neighborhood of 5 cc. throughout the remainder of the experiment.

Figure 23 is a chart which, at a glance, gives the details of this experiment. The leucocyte count rises somewhat during the whole course of perfusion, but the count of nucleated red cells makes no significant jump until the end of the first hour of perfusion when the blood is suddenly completely saturated with carbon monoxide. The condition of the bone-marrow at the close of the experiment is well illustrated in figure 24. The tissue contains many nucleated red corpuscles and these are freely bathed by the ink injection which is running through the marrow stroma in a very different manner from that shown in drawings of the marrows from previous experiments. The increase in nucleated red cells which occurred during the last half-hour of this experiment, we believe to have been due to the fact that the blood was wander-

ing freely through the marrow pulp—a state of affairs abnormal to the intact marrow tissue—and that young cells were displaced by the current.

At 4:18 an accident to the pump interfered seriously with the mechanical drive of the apparatus, and for the last 11 minutes of the perfusion and during the ink injection the tissue was being circulated less rapidly than would have been the case had the apparatus remained

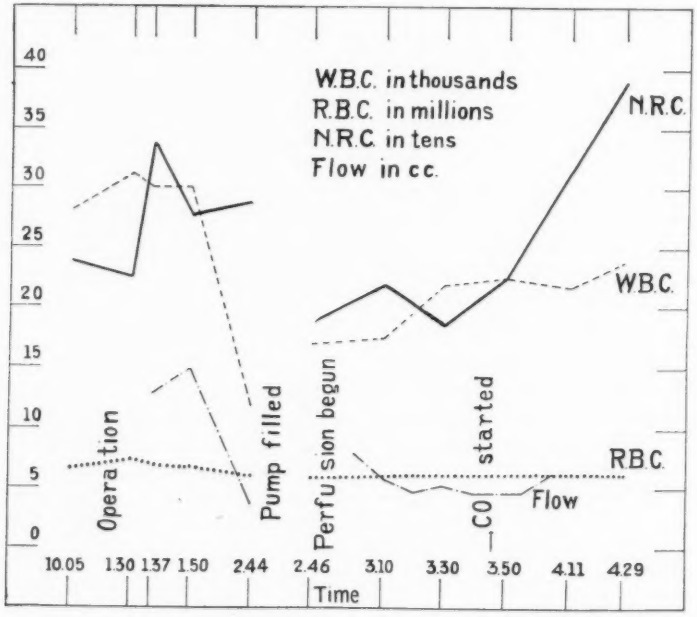

Fig. 23. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of flow in the perfusion experiment of November 24, 1917, dog K. K.

sound. Unfortunately, the time following this breakdown was occupied by efforts at repair, and a blood-flow measurement was not made. Figure 22, tracing 8, shows the great diminution in pressure which accompanied the regurgitation incident upon the break, and this was undoubtedly accompanied by a substantial fall in blood flow. It is certain that had pressure remained as in the antecedent period the marrow stroma would have been even more thoroughly flooded

with ink at the time of injection, and it is probable that both leucocytes and nucleated red cells would have risen more extensively in the final counts made at 4:29.

The experiment represents a further step in the process of which the last experiment (dog J. J., Nov. 3, 1917) possibly showed the very beginning. The picture is one of nucleated red cells leaving the marrow rather suddenly and as a result of a disintegrative process due to

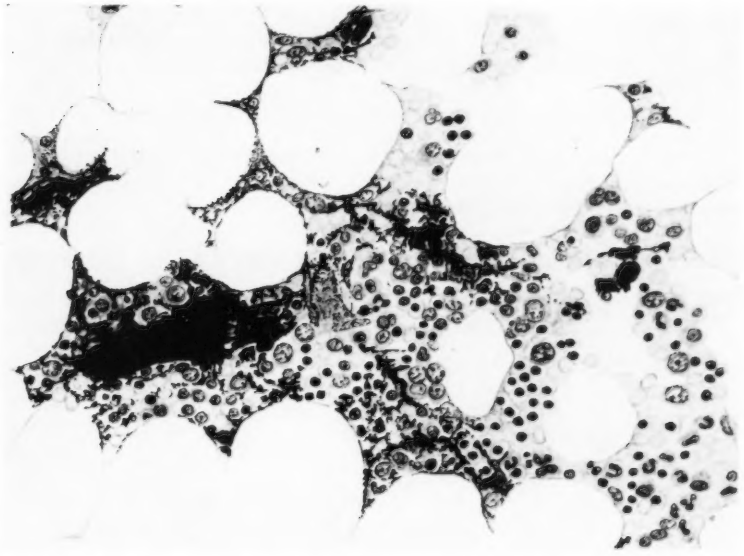

Fig. 24. Camera lucida drawing of the marrow injected at the close of the perfusion experiment of November 24, 1917, dog K. K. × 500.

the complete asphyxia of the tissue following saturation of the blood with carbon monoxide.

F. Perfusion under moderate pressure and moderate blood flow: Saturation of blood with carbon monoxide early in the perfusion.

Protocol 12. Dog H. H.; weight, 17.5 kgm.

As in a former case, this animal was bled several times prior to perfusion. The bleedings were accomplished under morphine anesthesia and were as follows:

August 8, 1917. Bled 780 cc. Infused 650 cc. of physiological salt solution during this hemorrhage.

September 6, 1917. Bled 775 cc. Infused 900 cc. of physiological salt solution during this hemorrhage.

September 27, 1917. Bled 750 cc. Infused 900 cc. of physiological salt solution during this hemorrhage.

November 3, 1917. Bled 420 cc. Infused 450 cc. of physiological salt solution during this hemorrhage.

November 10, 1917. *Perfusion.* Weight, 17.5 kgm.

8:25 a.m. 60 mgm. morphine sulphate subcutaneously.

9:50 a.m. 21.8 grams urethane by stomach tube.

10:04 a.m. Specimen 1, capillary blood: leucocytes 21,900; erythrocytes 5,872,000; nucleated red cells 0.

2:32 p.m. Isolation of the tibia completed in the usual manner, cannulas in place and preparations for filling the perfusion pump and starting perfusion completed.

Specimen 2, capillary blood: leucocytes 32,300; erythrocytes 5,613,000; nucleated red cells 48.

2:49 p.m. Figure 25, tracing 1, indicates the pressure relations in the artery of the left leg corresponding to the vessel in the right leg containing the inflow cannula for the perfusion. Mean pressure, 105 mm. of mercury.

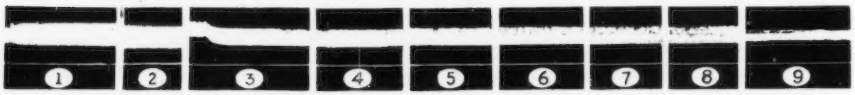

Fig. 25. Blood-pressure relations during the perfusion experiment of November 10, 1917 dog H. H.

2:54 p.m. Rate of blood flow through the tibial circulation, 15 cc. per minute.

Specimen 3, venous blood from tibia: leucocytes 30,900; erythrocytes 5,768,000; nucleated red cells 108.

3:00 p.m. Rate of blood flow through the tibial circulation, 15.6 cc. per minute.

3:02 p.m. Figure 25, tracing 2, pressure relations under the conditions of the dog's own circulation.

Animal bled 350 cc. into 75 to 100 mgm. of hirudin in not more than 20 cc. of physiological salt solution. Pump filled with this blood.

3:09 p.m. Perfusion started under the conditions shown in figure 25, tracing 3. This tracing shows the condition of affairs at the outset of perfusion with a prompt reduction in mean pressure to 100 mm. of mercury, at which level the pressure remained to the end of the experiment.

Specimen 4, blood taken from pump prior to passage through tibia: leucocytes 10,300; erythrocytes 6,377,000; nucleated red cells 0.

3:16 p.m. Rate of blood flow through perfused tibia, 9.1 cc. per minute.

3:17 p.m. Rate of blood flow through perfused tibia, 9.5 cc. per minute.

3:23 p.m. Specimen 5, blood taken from the pump after 14 minutes passage through tibia: leucocytes 11,400; erythrocytes 5,646,000; nucleated red cells 23.

3:25 p.m. Perfusing blood saturated with carbon monoxide.

3:27 p.m. Rate of blood flow through the perfused tibia, 12.1 cc. per minute.

3:28 p.m. Figure 25, tracing 4, pressure relations in the perfusion circulation.
3:37 p.m. Rate of blood flow through the perfused tibia, 11.3 cc. per minute.
3:38 p.m. Specimen 6, blood taken from pump after 29 minutes passage through tibia: leucocytes 12,700; erythrocytes 5,974,000; nucleated red cells 68.
3:39 p.m. Figure 25, tracing 5, pressure relations in the perfusion circulation.
3:47 p.m. Rate of blood flow through the perfused tibia, 11.6 cc. per minute.
3:53 p.m. Specimen 7, blood taken from the pump after 44 minutes passage through tibia: leucocytes 12,500; nucleated red cells 40.
3:57 p.m. Rate of blood flow through the perfused tibia, 12.4 cc. per minute.
4:06 p.m. Specimen 8, blood taken from the pump after 57 minutes passage through tibia: leucocytes 12,700; erythrocytes 5,491,000; nucleated red cells 114.
4:10 p.m. Rate of blood flow through the perfused tibia, 12.8 cc. per minute.
4:12 p.m. Figure 25, tracing 6, pressure relations at this time.
4:20 p.m. Rate of blood flow through the perfused tibia, 11.8 cc. per minute.
4:21 p.m. Specimen 9, blood taken from pump after 72 minutes passage through tibia: leucocytes 12,600; nucleated red cells 202.
4:22 p.m. Figure 25, tracing 7, pressure relations at this time.
4:30 p.m. Rate of blood flow through the perfused tibia, 11.9 cc. per minute.
4:38 p.m. Specimen 10, blood taken from the pump after 89 minutes passage through tibia: leucocytes 13,100; erythrocytes 6,349,000; nucleated red cells 367.
4:42 p.m. Rate of blood flow through the perfused tibia, 11.8 cc. per minute.
4:52 p.m. Rate of blood flow through the perfused tibia, 10.9 cc. per minute.
4:53 p.m. Specimen 11, blood taken from the pump after 104 minutes passage through tibia: leucocytes 11,200; nucleated red cells 386.
5:00 p.m. Figure 25, tracing 8, pressure relations at this time.
5:03 p.m. Rate of blood flow through the perfused tibia, 9.5 cc. per minute.
5:08 p.m. Specimen 12, blood taken from the pump after 119 minutes passage through tibia: leucocytes 10,100; erythrocytes 5,352,000; nucleated red cells 712.
5:12 p.m. Rate of blood flow through the perfused tibia, 5.6 cc. per minute.
5:13 p.m. Specimen 13, blood taken from the pump after 124 minutes passage through tibia: leucocytes 9,100; nucleated red cells 578.
5:14 p.m. Specimen 14, blood taken from the pump after 125 minutes passage through tibia: leucocytes 9,200; erythrocytes 5,165,000; nucleated red cells 662.
$5:14^{30}$ p.m. Figure 25, tracing 9, pressure relations during ink injection. On removal this bone weighed 70 grams and was thoroughly injected.

Comment. The subject of this experiment resembled dog J. J., described under section D, in that a period of blood loss preceded the marrow perfusion. Figure 26 shows that during the operation nucleated red cells appeared in the blood stream and were apparently increasing at 2:54 just prior to withdrawal of blood for the perfusion. The blood taken showed a great diminution in leucocyte count but no nucleated red cells were found in counting 2000 leucocytes in the film. Fourteen minutes after the beginning of perfusion, 3 nucleated red cells were found in counting 1500 leucocytes, giving 23 per cubic millimeter as the total at this time. Two minutes later the blood was

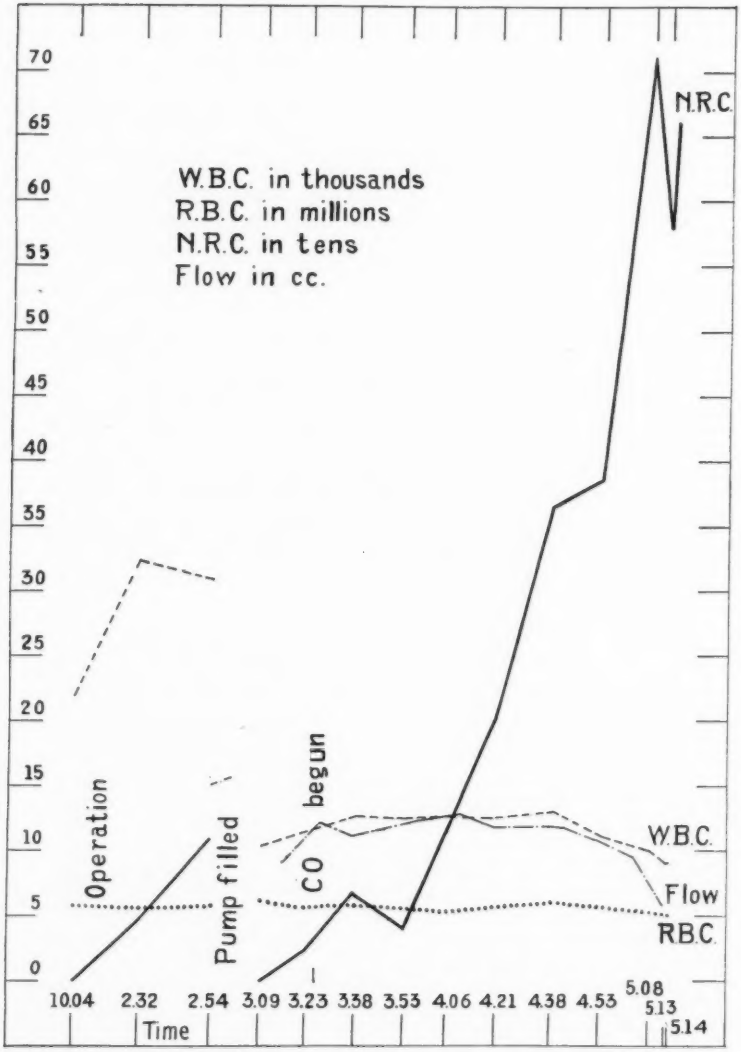

Fig. 26. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the rate of blood flow in the perfusion experiment of November 10, 1917, dog H. H.

saturated with carbon monoxide, and in 41 minutes the count of nucleated red cells rose suddenly and climbed rapidly to a high figure. This increase in nucleated red cells was not accompanied by a leucocytosis—indeed the leucocytes diminished in number toward the end of the experiment.

The blood flow under the conditions of the perfusion was somewhat less than under the animal's own circulation. It remained quite uniform until very near the end of the experiment when a rapid fall occurred. A point of considerable interest is the increase in blood flow which took place immediately after saturation of the blood with carbon monoxide. This occurred to a slight extent in the two preceding experiments, but was hardly worthy of comment. In the case under discussion the rise was from 9.5 cc. to 12.1 cc. per minute. On two other occasions, the details of which need not be given, the increase was even more marked. It is, of course, a familiar fact that local asphyxia results in vascular dilatation, and this case is noteworthy only for the fact that the reaction increasing the blood supply occurred within the blood-forming tissue.

Figure 27 shows the condition of a cellular portion of the marrow at the close of the experiment. The capillary outlines are beginning to break down and particles of ink are seen which have wandered out into the marrow stroma. Many nucleated red cells are present and, in view of their numbers, it is not surprising that the breakdown of the tissue resulted in a large accumulation of these young cells in the perfusing blood. Figure 28 is of particular interest. It displays a situation which can be found in practically all sections of fatty marrow but which was very prominent in this animal. The capillary shown is practically closed. It contains a few granules of ink and one red cell which has assumed an oblong shape very similar to the erythrocytes pictured by Krogh (5) in the case of collapsed capillaries in guinea-pig muscle. It is noteworthy that this closed capillary appears in marrow which has been subjected to prolonged asphyxia, and that these capillaries are particularly numerous in a bone-marrow which is hyperplastic as a result of several hemorrhages.

The experiment may be summarized as showing that, even in the presence of a slow blood flow, immature red cells may be washed from the bone-marrow in great numbers following the use of injurious agents.

IV. Injection experiments. The perfusion experiments which have been described show that the marrow tissue of the dog is not

diffusely circulated in the sense we accept for the spleen. The work of Mollier (23) has established the fenestrated character of the capillaries in this organ and has caused us to think of the circulation in the splenic pulp as a slow drift of blood through the tissue, which is, in

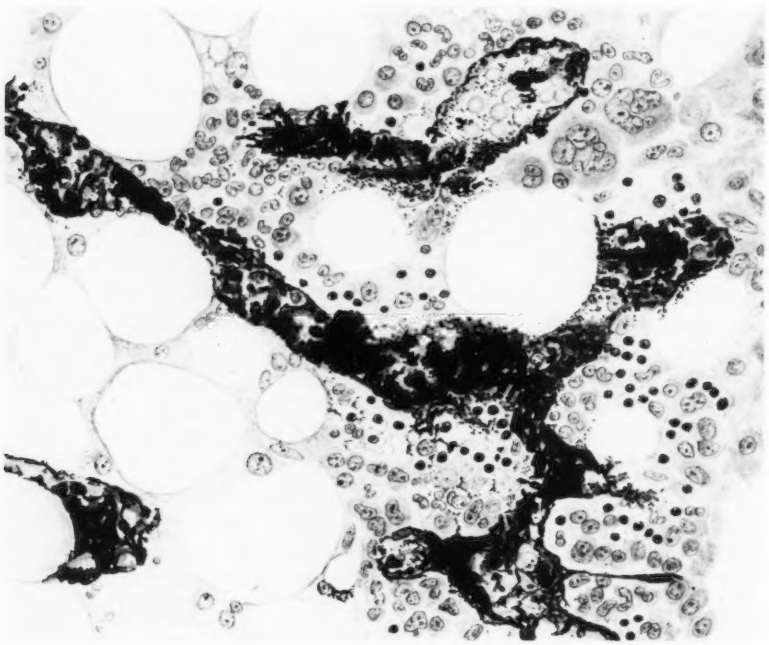

Fig. 27. Camera lucida drawing of the marrow injected at the close of the perfusion experiment of November 10, 1917, dog H. H. × 500.

some degree, controlled by the rhythmic contractions of the spleen. An open circulation in a contractile organ becomes, from a physiological point of view, closed, since the contractions should result in driving blood back into the vessels, thus preventing undue stuffing of the organ and eventual blockage of the circulation. Marrow, however, exists in a non-expansile bony case, and it is not surprising to find that the marrow capillaries remain intact even when subjected to extraordinary stress of blood-pressure and blood-flow. Many of the histological sections made following marrow perfusions show a definite endo-

thelial lining for the marrow capillaries. In other instances no such lining can be made out, and the injection mass is confined within irregular boundaries of young blood-cells. Failure to recognize an endothelial lining in such cases may be the expression of defects in

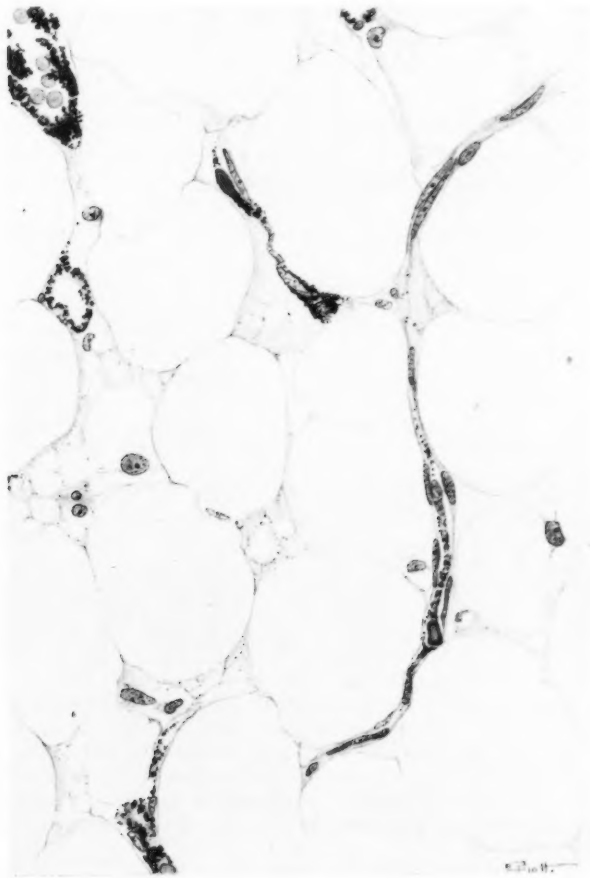

Fig. 28. Camera lucida drawing showing one large closed capillary typical of many seen in the marrow of dog H.H. (Perfusion experiment of November 10, 1917.) × 500.

histological technique, but it seems to us more probable that, during brief periods of intensely rapid growth, immature blood-cells overgrow the capillary endothelium and, for the time being, the blood current is bordered by marrow stroma.

In order to control and amplify this conception of the bone-marrow vasculature, numerous injections without antecedent perfusion have been made in dogs, cats and rabbits. The injection technique in these different species has been similar, the central idea being to inject in the living animal under pressure conditions simulating those existing in the animal at the moment of injection without preliminary interruption of blood flow, and with a fluid mass as little abnormal as can be devised. The results obtained have not varied in the different mammals employed.

1. The vascular relations in the marrow of the normal mammal.

Protocol 13. March 8, 1921. Rabbit Yb; weight 3 kgm.

9:20 a.m. 6 grams urethane by stomach tube.

10:00 a.m. A midline incision in the abdomen, followed by removal of the bladder and the lower part of the large bowel, exposed the bifurcation of the aorta. The left iliac artery was then freed and cleared for a distance of 1 inch just at its origin, all side branches being tied in this region. The artery was then ligatured at the distal end of the cleaned inch, a small bulldog clamp placed upon it at its origin from the aorta, and a cannula inserted pointing centrally. A ligature was then thrown around the aorta 1 inch above the iliac bifurcation but was not tied. The situation provided is shown diagrammatically in figure 29.

11:02 a.m. Figure 30, tracing 1, indicates the blood-pressure taken through cannula *B* by means of a membrane manometer, bulldog clamp *C* being temporarily removed.

11:05 a.m. Clamp *C* replaced. Cannula *B* connected with perfusion pump which had previously been arranged for injecting diluted dialyzed Higgins' India ink (cf. page 8). It is unnecessary to describe the manner in which the pump was set up to accomplish this injection. The reader will understand that the arrangement provided for injection of diluted India ink at body temperature and, as shown by figure 30, under pressure conditions normal for the animal at the moment. The arrangement also permitted an antecedent washing-out of the marrow vessels with Ringer's solution at body temperature and under physiological conditions of pressure. It was, however, found that this preliminary washing was unnecessary when the dilute injection mass previously described was employed, since this dilute mass itself served in the beginning as a wash for the marrow vessels.

11:11 a.m. Clamp *C* was removed, and the pump started. The pressure under which this change took place is shown in figure 30, tracing 2. The aortic ligature was at once tied and the right iliac vein cut. These manoeuvres resulted in shunting the injection mass into the right leg which at once became blackened. Figure 30, tracing 3, shows the pressure at which the injection was delivered.

11:19 a.m. Injection finished. Femur and tibia removed, and the shaft of the former opened to procure histological specimens. The tibia was cleared by the Spalteholz (4) method and, grossly, was apparently completedly injected.

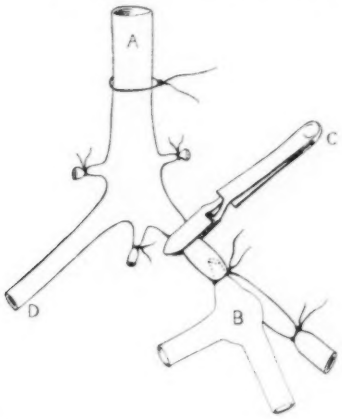

Fig. 29. Diagram showing the general anatomical relations governing injection experiments.

A, aorta, *B*, washout cannula, *C*, clamp, *D*, right iliac artery.

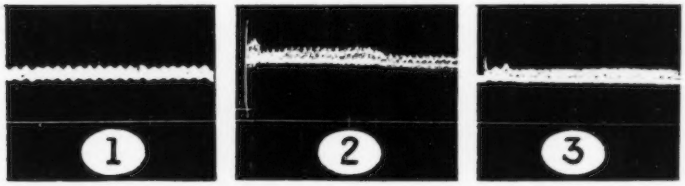

Fig. 30. Pressure relations in the injection experiment of March 8, 1921, rabbit Yb.

Comment. Figure 31 is a camera lucida drawing showing blood vessels in a relatively non-cellular portion of the marrow injected in this experiment. Traces of an endothelial lining can be made out and the injection mass is sharply marginated from the cell-bearing marrow.

Doan (24) has recently made an examination of the capillaries in the bone-marrow of the adult pigeon. He describes short "arterial capillaries" leading into wider "venous capillaries" and continues as follows:

There is little doubt that these extensively distributed, spacious, thin-walled venous sinusoids form normally the real functioning vascular bed of the marrow. These are the vessels which have been seen and described as the fundamental units of the bone-marrow by those who have written in this field. By most workers they are termed the venous capillaries. It would seem that venous sinus or venous sinusoid might be the more appropriate and desirable terminology, inasmuch as there are already two types of true capillaries in the marrow as recognized and interpreted in these observations.

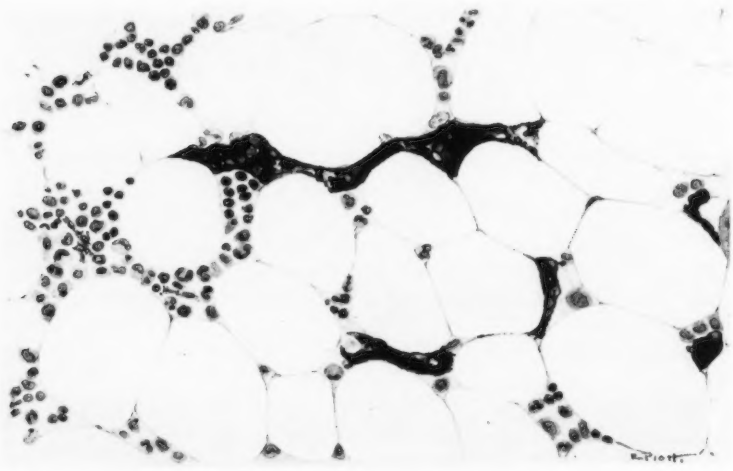

Fig. 31. Camera lucida drawing of the vessels in a relatively non-cellular region of the femoral marrow from rabbit Yb, March 8, 1921. × 500.

Figure 31 shows wide capillaries of the type thus described by Doan for the pigeon, and we agree that such vessels constitute the most important vascular elements in the marrow. From the point of view of function, however, it seems as if all vessels possessing an endothelial lining and nothing more, must share in receiving blood-cells as they pass from the marrow into the blood-stream.

In addition to the larger capillaries, Doan has described another set of capillaries in the starved pigeon, the existence of which has hitherto been unsuspected:

Many of these capillaries appear to have been non-patent and functionally dormant so far as the circulation is concerned. They are collapsed so that only a trace of fine ink granules reveals the presence of a potential lumen, the calibre

of which appears insufficient for the passage of even a single blood-cell element without difficulty. In an ordinary injection they are totally collapsed and are seen as septa surrounding the fat cell spaces. Toward the epiphyses there is a complete encircling of each fat space by these channels. They are seen to lead directly from the large venous sinusoids via typical conical openings and appear to be continuous with them. There is no break in the continuity of the endothelium which forms these slender channels from sinusoid to sinusoid. There is no extravasation at any point and the material injected follows these vessels everywhere. It is evident that these channels are closed in the sense that there is no extravasation or diffuse permeation of the tissue by the injected ink.

It has not been possible for us to satisfy ourselves of the existence of such a widespread system as this in the marrow of the normal mammal injected under the precautions we have described, although occasionally we have seen indications of its existence. Failure to obtain positive evidence of this large additional capillary plexus in our preparations is not surprising and in no way nullifies the possibility of its presence in the mammal. Doan was able to demonstrate these capillaries in the pigeon only after producing a rather complete marrow hypoplasia. Under such circumstances he found many vessels which had been entered by a fine stream of ink but which were at the moment closed to transmission of blood-cells. We have never made either perfusions or injections under comparable conditions of hypoplasia, and while we believe that our preparations show the full extent of the blood-carrying bed at the moment of injection and under pressure provided by the animal at the time, they are not necessarily equal to demonstrating the complete potential capillary bed which, as Doan has pointed out for the pigeon, probably does not disclose itself until the marrow is freed from developing blood-cells. As one examines our sections of injected marrow, he finds capillaries which vary widely in size and which frequently are so disposed that it is impossible to tell whether, at the moment, there is present a long single structure, or whether two separated capillaries of the venous type discussed by Doan are not joined by a widely open communicating capillary, which at a later period might well be closed. It is not difficult to appreciate that if the two moderate-sized capillaries at the bottom of figure 31 had, at the moment of injection, been connected by a channel between the fat cells which was wide enough to conduct a substantial amount of blood, the appearance obtained would not be far different from that of the larger, longer vessel at the top of the illustration. These capillaries are joined by a fine chain of ink granules indicating a rather completely closed connection at the moment of injection. This connecting channel does not

become larger in serial sections, and presents a fair indication of the extent to which our work verifies that of Doan.

In spite of final evidence upon the complete extent of the closed capillary bed of the mammalian marrow, we have obtained ample evidence that large capillaries are frequently practically closed to the passage of blood. Figure 28 shows such a capillary in the fatty marrow

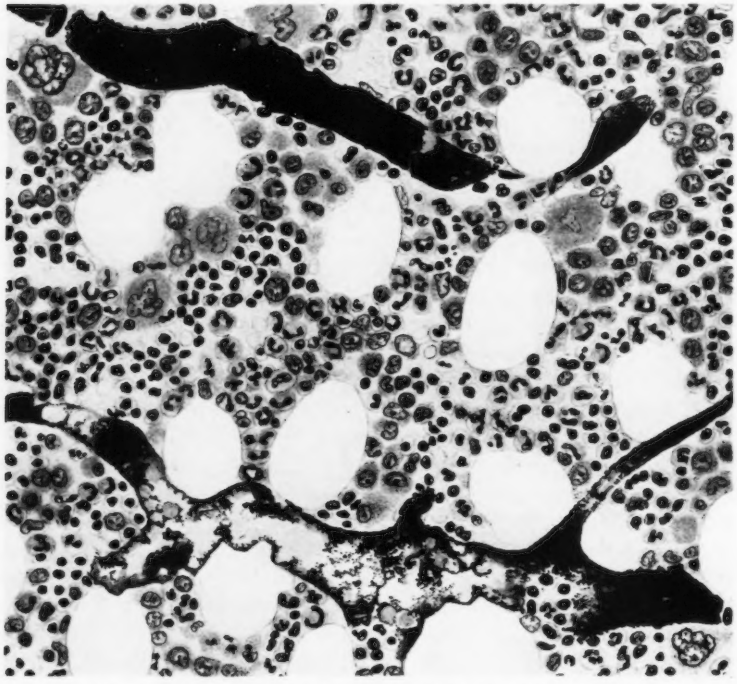

Fig. 32. Camera lucida drawing of a comparatively cellular marrow taken from a normal rabbit, no. 3, November 15, 1917. × 500.

near the center of the shaft of the tibia in an animal which had been bled several times in order to produce marrow hyperplasia. In this case the vessel shown is a long structure completely lined by endothelium, and containing a narrow current of ink and one somewhat deformed red blood corpuscle, which resembles the oblong corpuscles shown by Krogh (5) in his injections of the capillaries of voluntary

muscle in the guinea pig. The significance of such closed vessels, which were particularly numerous in this animal, will be considered in the later discussion.

Figure 32 is a high power drawing of the capillary relations in a more cellular marrow from a normal rabbit, injected under conditions similar to those described for the rabbit just preceding. The capillaries are large and are again sharply defined from the cell-bearing marrow. Occasional lining endothelial cells can be seen but the impossibility of tracing a continuous endothelium is manifest. The evenness of the

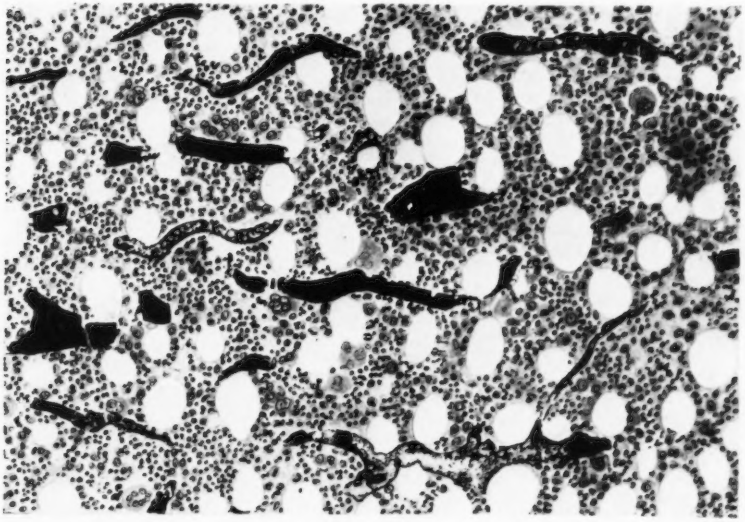

Fig. 33. Camera lucida drawing of the marrow region shown in figure 32, rabbit 3, November 15, 1917. × 180.

capillary outlines is to be noted and contrasted with the complete irregularity seen in figure 38, which represents the condition in hyperplastic marrow from a rabbit bled a number of times prior to injection. Figure 33, a low power drawing of the marrow region of figure 32, gives a good idea of the degree of vascularity observed in well-injected specimens of cellular marrow from animals in a normal state of equilibrium as regards blood formation.

2. The vascular relations in mammalian marrow, hyperplastic as a result of bleeding. In order to follow systematically the relations of

the capillaries to the extending marrow, cats and rabbits were bled small amounts at frequent intervals and finally injected with India ink. The following protocol illustrates an experiment of this type.

Protocol 14. Rabbit Xa; weight 3.3 kgm.

March 3, 1921. Specimen 1, capillary blood: leucocytes 15,000; erythrocytes 5,844,000; nucleated red cells 0.

Bled 20 cc. from ear vein.

March 4, 1921. Specimen 2, capillary blood: leucocytes 14,600; erythrocytes 4,929,000; nucleated red cells 0.

March 5, 1921. Specimen 3, capillary blood: leucocytes 15,200; erythrocytes 5,165,000; nucleated red cells 0.

Bled 26 cc. from ear vein.

March 7, 1921. Specimen 4, capillary blood: leucocytes 14,100; erythrocytes 4,380,000; nucleated red cells 14.

March 8, 1921. Specimen 5, capillary blood: leucocytes 13,700; erythrocytes 3,858,000; nucleated red cells 55.

March 9, 1921. Specimen 6, capillary blood: leucocytes 14,500; erythrocytes 3,706,000; nucleated red cells 29.

March 10, 1921. Specimen 7, capillary blood: leucocytes 9,200; erythrocytes 3,653,000; nucleated red cells 0.

Weight 3.1 kgm. Bled 25 cc. from ear vein.

March 11, 1921. Specimen 8, capillary blood: leucocytes 21,500; nucleated red cells 22.

March 12, 1921. Specimen 9, capillary blood: leucocytes 13,500; erythrocytes 4,098,000; nucleated red cells 41.

March 14, 1921. Specimen 10, capillary blood: leucocytes 16,100; erythrocytes 4,564,000; nucleated red cells 0.

March 15, 1921. Specimen 11, capillary blood: leucocytes 15,700; nucleated red cells 0.

Bled 25 cc. from ear vein.

March 16, 1921. Specimen 12, capillary blood: leucocytes 15,800; erthrocytes 4,275,000; nucleated red cells 0.

March 17, 1921. Specimen 13, capillary blood: leucocytes 14,300; erythrocytes 4,475,000; nucleated red cells 0; reticulated red cells 9.6 per cent.

The technique employed for staining the reticulated cells was that first described by Hawes (25) and later independently by Cunningham (26). The number of reticulated red cells in the blood of normal rabbits has been estimated by many different investigators. Thus, Pepper and Peet (27) declare them to be less than 2 per cent of the total number of red cells. Sappington (28) records counts upon a number of animals and does not find reticulated red cells until after administration of phenylhydrazine or after bleeding. Robertson (29) reports that, "In a large number of normal rabbits examined, the reticulated cells were found to vary for the most part between 10 and 20 per 1000

erythrocytes. Rarely they were as many as 30 or fewer than 5 per 1000. Only two animals showed less than 5 per 1000, one having a count of 3 and the other of 4."

This agrees with our own experience and it is surprising that Sappington found no reticulated cells in his animals prior to experimentation. The first count in our series resulted in finding 96 reticulated cells while counting 1000 red cells. This figure multiplied by 4475 (the number of thousands of red cells present) gives 429,600 as the number of reticulated cells per cubic millimeter. This number is 9.6 per cent of the total red cells per cubic millimeter and represents a decided increase over normal.

March 18, 1921. Specimen 14, capillary blood: leucocytes 15,200; erythrocytes 4,675,000; nucleated red cells 0; reticulated red cells 7.4 per cent.

Bled 40 cc. from ear vein.

March 19, 1921. Specimen 15, capillary blood: leucocytes 17,100; erythrocytes 3,281,000; nucleated red cells 0; reticulated red cells 7.9 per cent.

March 21, 1921. Specimen 16, capillary blood: leucocytes 15,100; erythrocytes 3,083,000; nucleated red cells 0; reticulated red cells 10.9 per cent.

March 22, 1921. Specimen 17, capillary blood: leucocytes 12,200; erythrocytes 2,906,000; nucleated red cells 0; reticulated red cells 8.4 per cent.

March 23, 1921. Specimen 18, capillary blood: leucocytes 12,600; erythrocytes 3,208,000; nucleated red cells 0; reticulated red cells 7 per cent.

March 24, 1921. Specimen 19, capillary blood: leucocytes 7,100; erythrocytes 3,034,000; nucleated red cells 0; reticulated red cells 3.9 per cent.

March 25, 1921. Specimen 20, capillary blood: leucocytes 6,700; erythrocytes 3,049,000; nucleated red cells 0; reticulated red cells 1.4 per cent.

March 28, 1921. Specimen 21, capillary blood: leucocytes 18,100; erythrocytes 3,296,000; nucleated red cells 18; reticulated red cells 5.7 per cent.

March 29, 1921. Specimen 22, capillary blood: leucocytes 22,700; erythrocytes 3,460,000; nucleated red cells 68; reticulated red cells 10.6 per cent.

Weight 2.8 kgm.

9:00 a.m. 5.5 grams urethane by stomach tube.

9:30 a.m. Operation for exposure of the iliac vessels begun and preparations for injection carried through as in the case of rabbit Yb (page 56).

10:12 a.m. Injection begun. Figure 34, tracing 1, indicates the pressure in the rabbit's circulation just prior to injection, and tracing 2, the actual injection pressure.

Comment. Figure 35 is a chart showing the cellular composition of the blood of rabbit Xa during 26 days in which there were five withdrawals of blood. The injection was made during a period of active delivery of young red cells, both reticulated and nucleated. Such crises are known to precede periods of increase in erythrocyte count and thus are indicators of active blood formation. Pencils of femoral

marrow from this animal were very thoroughly injected. On section, an area of fatty marrow about ½ inch in length was found in the middle of the shaft. Figure 36 is a low power drawing of the capillaries on the edge of this fatty marrow. The size and regular outlines of the vessels in this part of the marrow are not different from those seen

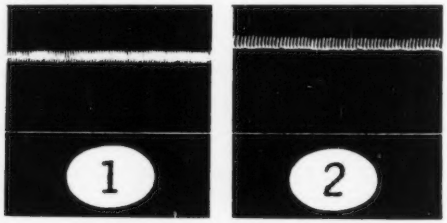

Fig. 34. Blood-pressure and injection pressure in rabbit Xa, March 29, 1921.

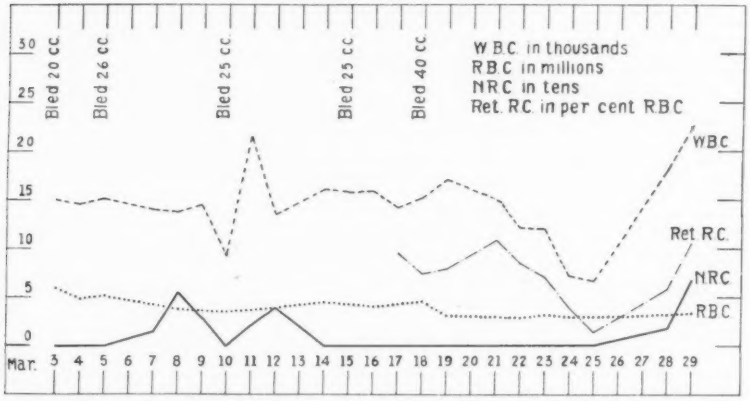

Fig. 35. Chart showing the leucocyte, erythrocyte and nucleated red cell counts and the percentage of reticulated red cells in rabbit Xa during the period between March 3 and March 29, 1921, the day of marrow injection.

in figures 31 and 32, which picture the capillaries in specimens of non-cellular and moderately cellular marrow from normal animals. When, however, in this animal, one moves to an area of highly cellular marrow, he finds a situation such as that depicted in figure 37. In this case a Y-shaped vessel, probably a small venule cut diagonally, occupies the center of a loose cellular tissue, through which, here and there, are

seen irregular masses of ink which fail to give the appearance of outlining capillaries. Examination of relatively few fields in such sections is, however, sufficient to convince the observer that the injected ink appears invariably in isolated masses and does not wander indis-

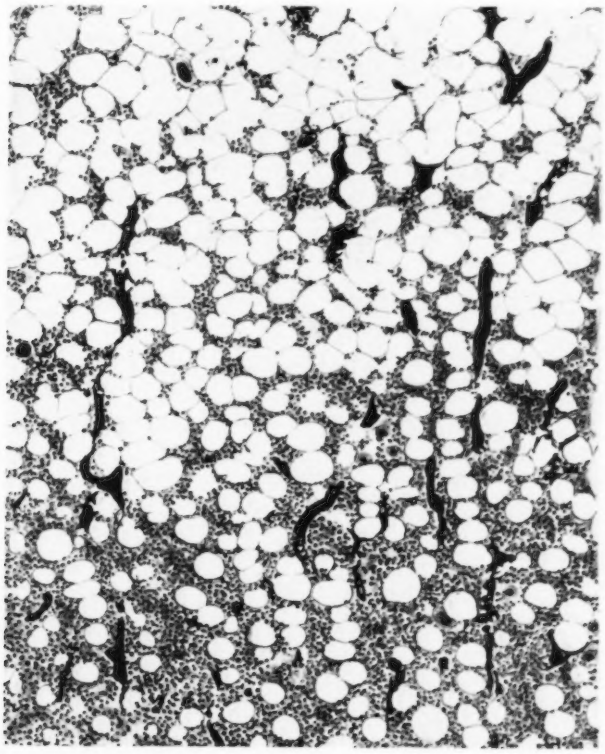

Fig. 36. Camera lucida drawing of a relatively non-cellular portion of the marrow taken from rabbit Xa, March 29, 1921. × 180.

criminately through the marrow stroma. The details of several capillaries under high power are seen in figure 38. All semblance of a definite vascular wall is gone. At the same time the injection mass is not free in the tissue but is held between columns of cells upon which it seems to border directly.

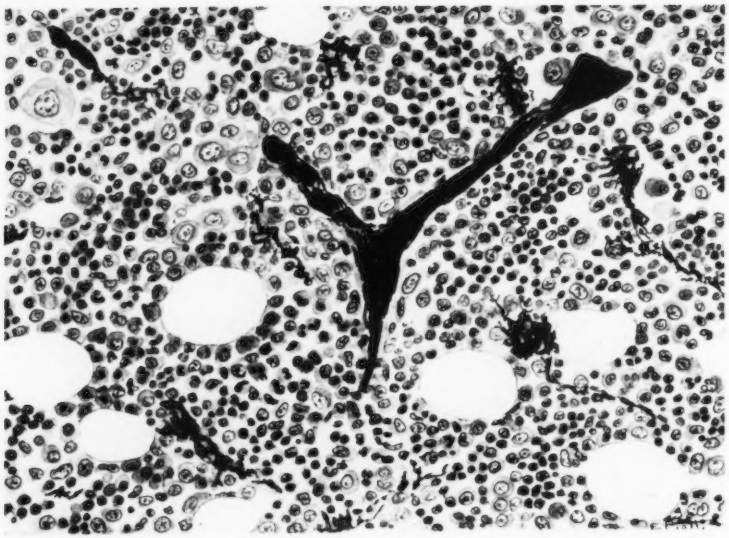

Fig. 37. Camera lucida drawing of hyperplastic marrow taken from rabbit Xa, March 29, 1921. × 380.

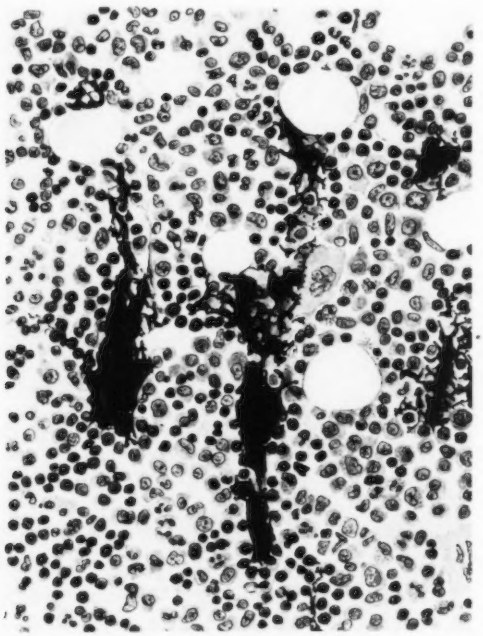

Fig. 38. Camera lucida drawing of capillaries in hyperplastic marrow. Note the extreme irregularity of outline and at the same time the failure of the injection mass to extravasate through the tissue. Rabbit Xa, March 29, 1921. × 500.

Appearances such as these lead to the conclusion that in marrow which is growing rapidly the tightly packed cells indent capillaries from all sides and, in the end, destroy areas of capillary wall so that the growing blood-cells eventually come in direct contact with the blood-current. It is our view that complete integrity of wall represents the normal state of the marrow capillaries and the condition to which these vessels will ultimately return, provided opportunity is given. Even under normal circumstances, mature red cells must constantly be passed from the marrow tissue into the blood-current, impelled in all probability by the growth of younger forms in their neighborhood. This process in the normal animal is so nicely ordered as to interfere very little with the circulation. When, however, the need for blood-cells suddenly becomes great, growth of cellular marrow proceeds faster than removal of fat, and capillary outlines, offering as they do the most yielding direction for growth, become indented and even abolished by the tightly packed cells. This is the condition in rabbit Xa, pictured by figure 38, and under such circumstances it is not surprising that young blood-cells are found in the blood-stream—the situation presented by this animal at the moment of injection. The reader will recall the result obtained in the perfusion experiment upon dog D. D. D., an animal in extraordinarily active blood-cell growth at the time of perfusion. The large extrusion of nucleated red cells which occurred during this perfusion was the result of forcing blood through a tissue in a condition such as that shown in figures 37 and 38.

Further evidence upon the vascular relations in hyperplastic marrow is given by the following experiment upon a cat.

Protocol 15. Cat Xd; weight 3.5 kgm.

March 25, 1921. Specimen 1, capillary blood: leucocytes 18,600; erthyrocytes 6,760,000; nucleated red cells 19.

Bled 46 cc.

March 28, 1921. Specimen 2, capillary blood: leucocytes 19,800; erythrocytes 4,490,000; nucleated red cells 20.

March 31, 1921. Specimen 3, capillary blood: leucocytes 27,200; erythrocytes 4,570,000; nucleated red cells 190.

April 2, 1921. Specimen 4, capillary blood: leucocytes 32,700; erythrocytes 5,705,000; nucleated red cells 0.

April 5, 1921. Specimen 5, capillary blood: leucocytes 35,200; erythrocytes 5,140,000; nucleated red cells 0.

April 7, 1921. Specimen 6, capillary blood: leucocytes 40,500; erythrocytes 5,537,000; nucleated red cells 0.

Bled 25 cc.

April 8, 1921. Specimen 7, capillary blood: leucocytes 23,000; erythrocytes 4,784,000; nucleated red cells 0.

April 12, 1921. Specimen 8, capillary blood: leucocytes 23,200; erythrocytes 7,110,000; nucleated red cells 0.

Bled 26 cc.

April 15, 1921. Weight 3.8 kgm. Specimen 9, capillary blood: leucocytes 33,400; erythrocytes 6,244,000; nucleated red cells 50.

9:56 a.m. 9.5 grams urethane by stomach tube.

11:50 a.m. Exposed the bifurcation of the aorta and placed a cannula pointing centrally in the middle sacral artery. A ligature about the aorta above the bifurcation was tied at 12.10 after the injection had been started under the pressure shown in figure 39, tracing 2. Tracing 1 is the animal's own pressure taken through the same cannula a few moments before. Section of the iliac veins allowed the ink injection to run out freely. The use of the middle sacral artery permitted an excellent injection of both legs.

Comment. The chart shown in figure 40 summarizes the preparation of this cat for injection. It will be seen that the nucleated red cells

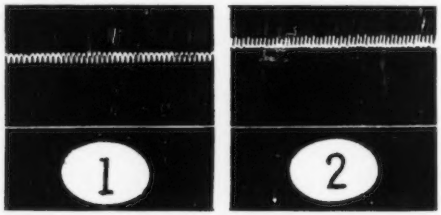

Fig. 39. Blood-pressure and injection pressure in cat Xd, April 15, 1921.

have again begun to move into the circulation on the day the injection was made. A reticulated red cell count on the same day gave a high figure—42 per cent. Figure 41 is a high power drawing of the marrow from this animal. The irregular outline of the large capillary is characteristic of the conditions found in rapidly growing marrow. An endothelial lining cannot be seen. Again, however, the injection mass does not move into the marrow pulp but remains within bounds. Here and there in this drawing as well as in figure 38, which portrays the capillaries in hyperplastic rabbit marrow, individual marrow cells seem to be standing directly in the blood current. Their appearance in such positions does not necessarily mean they are being washed away since tongues of cells growing at right angles to the plane of section would give the same appearance. Growth encroachment upon a marrow capillary must occur from all sides and the development of irregularity of outline and the appearance of marrow cells, even in the midst of the injected ink, does not mean that the cells are as yet detached from their site of growth.

3. The vascular relations in mammalian marrow after injection of saponin. In 1906, Bunting (30) performed a series of suggestive experiments upon the action of ricin and saponin. Rabbits were used and the effects of saponin administered intravenously are described as follows: "destruction of red blood cells in the circulation and to a less extent of the leucocytes, a destruction of cells in the marrow and in particular an injury to the capillary walls resulting in extensive

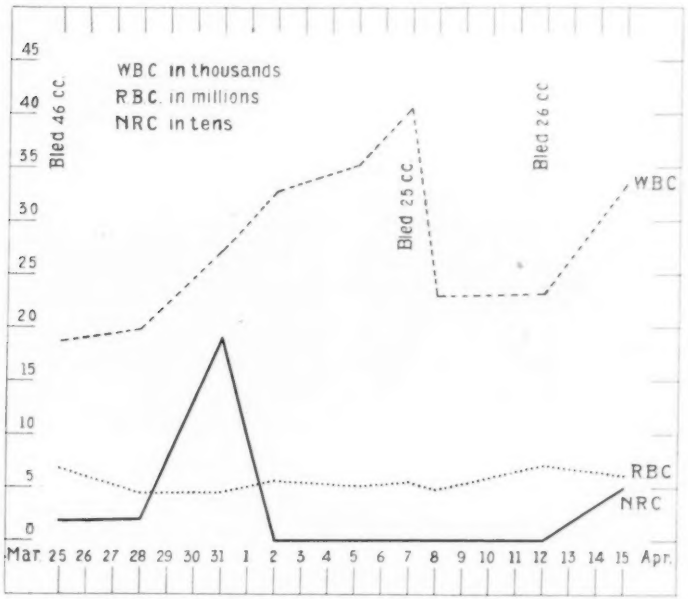

Fig. 40. Chart showing the leucocyte, erythrocyte and nucleated red cell counts in Cat Xd during the period between March 25 and April 15, 1921, the day of marrow injection.

haemorrhages. The circulating blood shows a reduction in the red-cell count, and sharp nucleated red-cell crises, and later, pathological changes in the erythrocytes." In another section, Bunting speaks of the diffuse marrow haemorrhage induced in acute poisoning by saponin in the following terms: "The last [haemorrhage] is the most striking lesion and was present in all the marrows studied. It seems somewhat remarkable that there should be such extensive haemorrhage

in the marrow when other organs of the body are practically free from it. There are no macroscopical haemorrhages such as are the usual lesions in ricin poisoning, nor are microscopical haemorrhages frequently found. The delicate capillaries of the marrow seem especially susceptible to the poison." With this description we are in agreement. The most pronounced effect of saponin upon the marrow consists of an opening of the marrow stroma to the blood current. A circulation which has been confined to very definite channels becomes diffuse and, accompanying this change, there is the appearance of nucleated

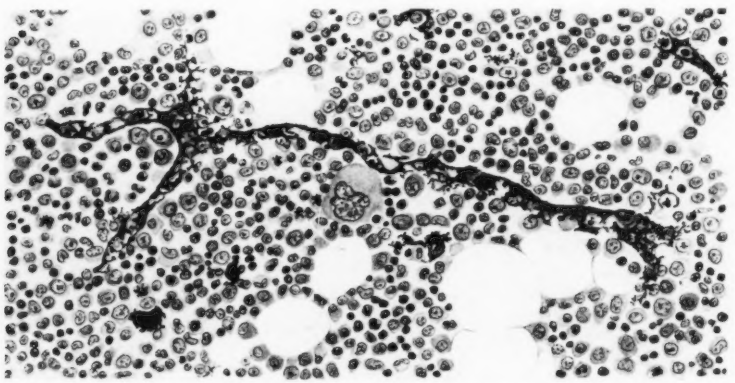

Fig. 41. Camera lucida drawing of a large capillary in hyperplastic marrow from cat Xd, April 15, 1921. × 500.

red cells in the peripheral blood. An illustrative experiment is as follows:

Protocol 16. September 24, 1920. Cat 12 C; weight, 3.3 kgm.

2:05 p.m. Specimen 1, capillary blood: leucocytes 24,500; erythrocytes 9,582,000; nucleated red cells 25.

2:10 p.m. 6.6 mgm. saponin administered intravenously.

3:30 p.m. Specimen 2, capillary blood: leucocytes 16,000; nucleated red cells 112.

4:15 p.m. Specimen 3, capillary blood: leucocytes 23,200; nucleated red cells 441.

5:15 p.m. Specimen 4, capillary blood: leucocytes 76,100; nucleated red cells 609.

5:45 p.m. 6.25 grams urethane by stomach tube.

6:25 p.m. Between 5:45 and 6:25 the dissection of the iliac arteries, and the placing of ligatures, cannula and clamp, as shown in figure 29, were accomplished and the blood-pressure tracing, shown in figure 42, tracing 1, was taken.

6:30 p.m. Specimen 5, capillary blood: leucocytes 163,800; erythrocytes 5,600,000; nucleated red cells 2948.

Following the taking of this specimen, Ringer's solution was pumped through the leg and was followed by India ink injection, the two fluids being introduced under the pressure shown in figure 42, tracing 2. This pressure was 190 mm. of mercury as against 130 mm., the pressure maintained by the cat. The high injection pressure was necessitated by difficulty in getting the Ringer's solution to flow under pressure comparable to that maintained by the animal.

Figure 43 is a drawing of the bone-marrow from this animal. No definite blood-vessels are visible and the marrow has been depleted of growing cells, their places being taken by erythrocytes. The injected

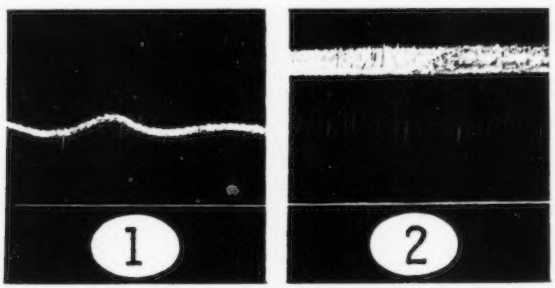

Fig. 42. Blood-pressure and injection pressure in cat 12 C, September 24, 1920.

ink has wandered diffusely through the tissue—an expression of the complete loss of vascular integrity following the saponin. Figure 44 is a higher power drawing of the marrow of a rabbit injected after the administration of saponin. In this case, the injection pressure was identical with that of the animal, and again it is seen that the India ink has spread diffusely through tissue which has suffered a high degree of cellular depletion. A brief summary of this experiment is as follows:

Protocol 17. March 22, 1920. Rabbit 8; weight, 2.6 kgm.

9:25 a.m. Specimen 1, venous blood: leucocytes 11,800; erythrocytes 5,182,000; nucleated red cells 0.

9:40 a.m. 5.2 mgm. saponin administered intravenously.

11:25 a.m. Specimen 2, venous blood: leucocytes 11,900; nucleated red cells 2535.

2:36 p.m. Specimen 3, venous blood: leucocytes 58,100; nucleated red cells 5810.

2:49 p.m. Injection.

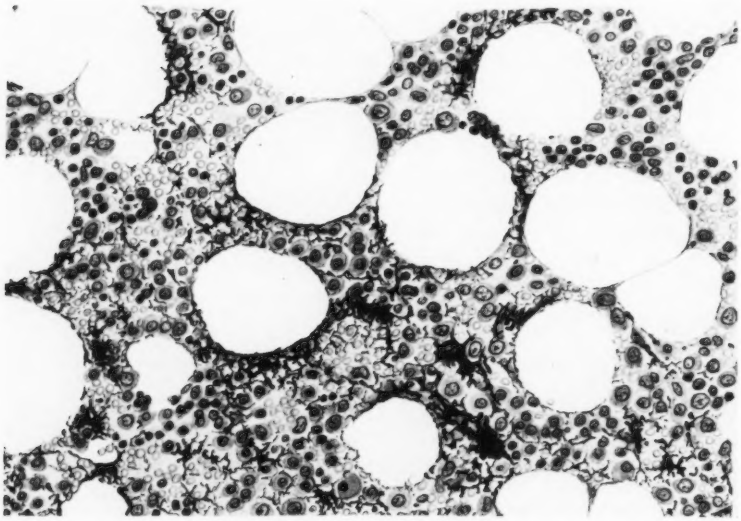

Fig. 43. Camera lucida drawing of the injected bone-marrow of cat 12 C, following intravenous administration of saponin, September 24, 1920. × 380.

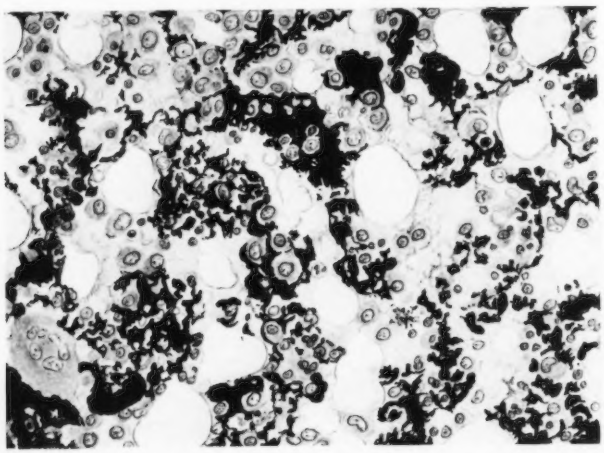

Fig. 44. Camera lucida drawing of the injected bone-marrow of rabbit 8, following intravenous administration of saponin, March 22, 1920. × 380.

Comment. In both of these animals (cat 12 C and rabbit 8) there was a large escape of all sorts of young cells from the marrow into the circulation. Indeed, except for the absence of fully developed megacaryocytes which are filtered out in the lung capillaries, blood films following saponin injections are very similar to films made from normal marrow. It is apparent in the two protocols which have been presented that nucleated red cells appeared in the blood stream before the leucocyte count began to rise. Bunting (31) has described a very orderly disposition of the erythrogenetic cells of the bone-marrow, finding them in groups with the youngest cells in the center and the more and more mature cells on the outer margins nearest the capillaries—a description with which Selling (32) agrees. Occasionally, we have felt that such an organization exists but it is very difficult to obtain constant assurance of it. Whether or not the colony arrangement of the erythrogenetic marrow is true—and it offers a plausible explanation for the fact that under normal circumstances the peripheral blood does not contain abnormally young red cells—it is of interest that in the case of saponin disintegration of the bone-marrow and that in certain of our perfusion experiments nucleated red cells have been washed from the marrow prior to an increase in the leucocyte count. One explanation for this phenomenon would be closer proximity to the circulating blood.

V. DISCUSSION

During the past fifty years hematology has, in the main, been a science of identification and classification, and this phase of the subject is by no means finished today. Sabin (33) has recently reviewed the progress which has been made, and we need only concern ourselves with such aspects of the questions concerning blood-cell origin as are related to our own experiments—experiments which have been designed to disclose certain facts upon the manner of delivery of blood-cells from the bone-marrow to the blood-stream.

If one turns back to the first appearance of blood formation in the embryo chick, one finds that red blood-cells are first developed intravascularly, arising from the endothelium of the blood vessels (34), (35). This vascular endothelium has, in its turn, arisen from undifferentiated mesodermal cells. Maximow (36) in his studies of mammalian embryos, while agreeing that the first stages of blood formation are intravascular, refers the inception of blood formation in all locations to an undifferentiated basophilic cell, called by him a "lymphocyte,"

and contends that when blood-cells arise from endothelium they do so as a result of the production of such "lymphocytes" from vascular endothelial cells, and that this new generation then goes on to blood formation. It is unnecessary for us to pursue the details of a histological controversy since we are interested in but one fact upon which it is apparent the best authorities agree; namely, that in the earliest stages of embryonic life blood is formed inside blood-vessels and that this may occur in capillaries which are conducting a current of blood.

Blood formation, beginning in this way in the embryonic mesoderm, is next seen in the liver and the spleen, and eventually begins to appear in the bone-marrow. We are not concerned with the problem except in the last locality, which is destined to become the source of red blood-cells and leucocytes throughout the life of the animal. Danchakoff (37) comments upon the development of hematopoiesis in the bone-marrow in the following terms:

> Finally, I have to emphasize the striking analogy seen in the general conditions which accompany the development of every new erythro-granulopoietic organ: an abundance of food supply and the localization distant from any rapidly growing organs; these conditions are met with both in the yolk sac and in the cavities of the bones and seem to be so essential that in reptiles which have no extremities, hematopoiesis finds its localization in the cavities of the vertebra, thus developing a hematopoietic organ in the form of a whole series of isolated centres (Danchakoff). Even the localization of hematopoiesis in the liver of mammals, which have lost the yolk in their eggs, corresponds in the highest degree to the conditions cited and which, at this time, the embryo offers.

When one considers the loose and semifluid organization of blood-forming tissue and the fact that the adult element formed within it must enter the circulating blood without dislocation of young forms, and when one considers that this process must take place in animals whose manifold activities result in great variations of blood-pressure and blood-flow, it becomes clear that isolation of the tissue within a bony case offers decided physiological advantages which may be added to the statement of Madame Danchakoff.

Maximow (38) has given the clearest account of the histogenesis of the bone-marrow in mammals and believes that red blood-cells are formed entirely extravascularly in this tissue. He describes the origin of red blood-cells in the marrow, and, indeed, of all the blood-cells, from undifferentiated mesenchymatous elements exactly similar to the large basophilic cells called "lymphocytes" in his earlier work upon the first phases of blood formation in the embryo. One section of

Maximow's (38) paper bears so directly upon our own experiments as to merit complete quotation:

The second question, upon the manner and way in which the ripe erythrocytes get into the blood stream, as has been said before, is not easy to answer. We have already seen that the lymphocytes [here he means the large basophilic cells which are the precursors of the blood-cells and not the lymphocytes of usual hematological terminology] enter the blood stream through active migration. Similarly, the granulocytes described later are capable of ameboid movement. But how do the inert erythrocytes reach the blood stream? By careful study of preparations under high magnification a positive opinion on this question can be formed.

The development of the lymphocytes to erythroblasts appears always in the immediate neighborhood of the blood vessels, which is readily understood, since the blood-forming tissue intervenes in relatively small strips between the endochondral bony trabeculae and the numerous blood vessels. The groups of erythroblasts lie, for the most part, very closely outside the endothelial wall of the blood vessels and occasionally arch the vascular membrane inward. This statement is especially applicable to the erythroblastic colonies consisting of ripe erythroblasts with pyknotic nuclei. The cellular composition of the groups may, however, be very variable.

At this time the endothelium of the blood vessels in the centre of the diaphysis, where blood formation is occurring, undergoes very special changes. While the endothelium in the first stages of marrow formation, in the lymphoid marrow, was still primitive and developing it showed cells multiplying mitotically, and the same condition was noticeable later in the vascular endothelium in the regions of ossification and in the zone of spreading lymphoid marrow. Now, however, the endothelium in the central older blood-forming portion of the marrow space becomes thinner and more delicate. Mitoses are no longer found. The cells flatten markedly, the nuclei become pallid, poor in chromatin, withdraw further and further from one another, and, in many places in cross or longitudinal sections, one sees wide sinusoidal vessels bordered by an external thin pale line, the cross-section of the endothelial membrane, the nuclei of which have been missed in the section. Outside this line lies the edematous, pale, amorphous interstitial substance, the marrow tissue, rich in wandering cells and blood-cells of different types, with occasional pale connective tissue cells between them. That the groups of erythroblasts lie with especial predilection immediately against the endothelial wall of the capillaries and arch the thin membrane inward, I have already said.

Now one sees by sufficient examination, that in the region of the erythroblastic colonies, where in the young tissue one may also find ripe non-nucleated erythrocytes, the thin endothelial membrane here and there loses its continuity—it appears torn and sieve-like. The very pale walls of such crevices are not easily seen on account of the cellularity of the tissue. Through the openings in the endothelial membrane blood plasma passes into the tissue, loosens the thick groups of cells and gradually washes the ripe erythrocytes from the tissue into the vessel lumen. Beside the young non-nucleated but still irregularly shaped erythrocytes, a certain number of nucleated normoblasts reach the blood stream. To be sure, the largest part of the normoblasts have pyknotic nuclei, which on

passage into the vessels appear to be shed immediately. Inside the vessels one sees, in such cases, in addition to regularly formed old cells, irregular, young, non-nucleated erythrocytes, normoblasts with pyknotic nuclei, some of which have been fixed at the moment of nuclear extrusion, and free, naked, extruded nuclei. Megaloblasts get inside the blood vessels only in very small numbers and complete their development quite normally in the blood stream. Lymphocytes of different types in the same areas only occasionally pass through the open endothelial wall into the blood. It is noteworthy that this never seems to happen with cells of the granular series.

Naturally adult erythrocytes extravasate into the tissue with the blood plasma. One always sees a limited number of such erythrocytes in the groups of erythroblasts emptying into the blood stream.

After a time the thin endothelial membrane, which consists of contractile protoplasm, can again close and the vessel wall regains its continuity.

Insofar as our knowledge of the organization of the bone-marrow in adult animals has gone, it is not essentially different from Maximow's description of the tissue in the embryo. Red cells are apparently formed outside the blood-stream and enter the moving current as a result of growth pressure. It will be noticed that we have not declared for the extravascular origin of the erythrocytes, but have simply said that they arise outside the blood-stream. The work of Doan, to which we have already referred, has disclosed a most extensive set of capillaries in the marrow of birds which has heretofore been unsuspected but which has, perhaps, been responsible for a certain amount of the confusion existing as to the relation of the developing erythrocytes to the capillaries. It will be remembered that Doan was able to inject these extra-capillaries in pigeons whose marrow had been rendered hypoplastic by starvation.

From a physiological point of view the marrow cavity of a bone is occupied by five elements: *a*, marrow framework—an extremely delicate reticulum; *b*, fat cells; *c*, developing and abult blood-cells; *d*, blood-vessels and circulating blood; and *e*, intercellular fluid—water. Of these elements the first is incompressible and comparatively immovable. The second—the fat cells—are incompressible, comparatively immovable, and only capable of yielding space through the slow process of absorption and possibly to a slight degree through deformation. The developing and adult blood-cells are incompressible, and, if growing actively, must obtain room by crowding out interstitial fluid and crowding into the capillaries, thereby encroaching upon their lumina. The fourth and fifth elements in our physiological system, while incompressible, are at the same time readily movable, in the sense that the amount of blood inside the vascular system of the marrow

may vary and that the amount of interstitial fluid may likewise change, both as a result of removal by the blood-vessels and by lymphatic pathways.[4]

It seems to us possible that Doan's success in injecting a capillary system, the existence of which has not been suspected in ordinary bone-marrow, depends upon the fact that starvation has resulted in removing fat cells and developing blood-cells. The space formerly occupied by these elements is filled with water and with blood inside the vessels. If, at this time, one makes an injection under a fair degree of pressure, the movement out of the marrow along lymphatic routes of even a small amount of water would permit the opening of capillaries heretofore closed to the entrance of blood. The capillary system described by Doan is continuous with the system normally carrying blood, but according to his description: "Many of these capillaries appear to have been non-patent and functionally dormant so far as the circulation is concerned. They are collapsed so that only a trace of fine ink granules reveals the presence of a potential lumen, the calibre of which appears insufficient for the passage of even a single blood-cell element without difficulty." The marrow tissue of birds thus apparently possesses vessels in addition to those being circulated at the moment. At first sight, this seems merely a repetition of a state of affairs already familiar for other tissues. It seems to us, however, to open another possibility; namely, that throughout the marrow stroma capillaries exist closed off from the actual circulation and given over to the formation of blood, as is seen in the most primitive embryonal conditions. Our ability to identify capillary walls in bone-marrow with any reliability at all depends upon injections. Closed-off vessels in the thickly cellular marrow tissue could not be identified in ordinary sections, and might readily, in such positions, constitute foci of blood formation quite similar to those seen in the earliest blood-forming tissue.

Many investigators have commented upon the character of the blood-vessels in the bone-marrow of adult animals and upon the relations of the circulating blood to the marrow tissue. Hoyer (39), in 1869, in the year following Neumann's discovery of erythrogenesis in the bone-marrow, injected the marrow of the rabbit and could demonstrate no

[4] We know of no work upon the lymphatic system of the bone-marrow, and, in connection with the lymphatic pathway, merely assume that some of the interstitial fluid probably passes out along the sheaths of the entering blood-vessels. Such drainage must be slight and quite unimportant compared with the removal of fluid by the blood-stream.

walls to the capillaries. Rindfleisch (40), writing in 1880, was of the same opinion. He also made injections of the marrow vessels in both guinea pigs and rabbits, and reported: "The veins of the red bone-marrow as well as a large part of the capillary vessels possess no true wall. The arteries have a weak lining which continues into the beginning of the capillary bed and is then lost."

In 1880, Bizzozero and Torre (41) observed fully walled capillaries in the marrow of starved birds, and Denys (42), in 1887, reaffirmed this finding and described the formation of red blood-cells within the capillaries. Van der Stricht (43), in 1892, in an extensive and frequently quoted paper upon blood formation in both birds and mammals, believed the situation in the marrow differed in the two species.

> The structure of the bone-marrow of mammals differs markedly from that of birds. This difference resides in the character of the venous capillaries. In birds these vessels possess an endothelial wall continuous throughout their entire extent. The blood circulating in these vessels does not come in contact with the erythroblasts developing and multiplying inside the hematopoietic capillaries. The different types of white cells pass across the endothelial wall by diapedesis but multiply outside the vascular system.
>
> In the bone-marrow of mammals the walls of the venous capillaries are discontinuous. The blood goes through these openings, and wanders freely into the meshes of the neighboring adenoid tissue. Inside the meshes of this tissue the erythroblasts and leukoblasts divide and develop.

The semi-diagrammatic drawings in Van der Stricht's paper make these statements quite vivid and he applies them to mammals in general. His illustrations of mammalian marrow, however, deal with the rabbit alone, and he did not use injections to identify the actual limits of the vessels through which blood was flowing. Our own experience has shown us that, lacking properly made *in vivo* injections, one might readily conclude that the capillaries in rabbit marrow are fenestrated. Certainly one must arrive at this conclusion if inability to trace a continuous endothelial lining for sectioned vessels is considered certain evidence for its absence during life. In the capillaries of the normal rabbit it is only occasionally that one can trace the extremely delicate lining endothelium, even when aided by an injection mass, and without this the search would seem quite futile. Reference to figures 31, 32 and 33, which show the injected capillaries of the normal rabbit, will indicate the impossibility of tracing a continuous endothelium, and, at the same time, display the entire incorrectness of Van der Stricht's statement that the circulating blood wanders freely through the marrow tissue.

Brinckerhoff and Tyzzer (44) in their studies of uninjected rabbit marrow remark: "In places, the blood stream is not confined within endothelial walls, but wanders through channels in the reticulum and the masses of cells."

In contrast to these views, Langer (3) made injections of mammalian bones and found the capillaries to be intact structures, and Minot (45) remarks that, "Certain investigators assert that the cavities of the blood-vessels are in direct open connection with the spaces of the mesenchyma, but, so far as I know, the conclusive proof of the correctness of this statement is lacking."

Without further quotations from the literature, it is safe to say that where opinions have been expressed upon the character of the marrow vessels in mammals they have been predominately in favor of a non-closed system. We believe that this opinion has arisen from the following causes: *a*, imperfect microscopical preparations; *b*, failure to realize that the appearance of the marrow may vary with the rate of growth of blood-cells at the moment of examination; *c*, failure to consider the effect upon the tissue of an open vascular system; and *d*, failure to consider the manner in which erythrocytes reach the circulating blood. Our own experiments, both perfusions and injections, have been designed to cope with these four elements in the problem. The facts which our work brings out will be understood much more readily if we first review the different lines of evidence dealing with the manner in which red blood-cells reach the circulating blood.

We have already pointed out that movement of red blood-cells from the marrow can occur as a result of three possible causes, *a*, ameboid migration, *b*, growth pressure, and *c*, disintegration of the marrow tissue. The first of these causes may be dismissed at once. Ameboid movement has unquestionably been seen in very immature red cells, but never in erythrocytes. The second, growth pressure, is of great importance and was first offered as an explanation of red-cell delivery by Rindfleisch (46) in 1880. He described the peculiar position of the blood-forming tissue in a bony case and held that growth under these circumstances could force red cells into the blood-vessels, which, as we have seen, he believed to be wall-free. We have already referred to Maximow's (38) description of a similar method of cell delivery in the first phases of the life of the bone-marrow. Maximow pictures capillaries with perforated walls during periods of active cell delivery, but adds a very significant sentence to the effect that these openings may later on become closed. Bunting's (47) description

of the process of cell delivery is identical with that of Rindfleisch, except that he does not definitely commit himself upon the question of the final character of the marrow capillaries—whether they are perforated or intact. None of these expositions of the "growth-pressure" theory is accompanied by satisfactory experimental evidence. They consist of deductions based upon the histological appearance of the marrow, unattended by observations upon the functional position of the tissue at the moment of marrow removal.

For many years it has been a familiar fact that in anemia nucleated red cells are found in the peripheral blood, and hematologists, particularly those interested in the clinical aspects of the subject, have been accustomed to reason upon the cell-forming activity of the marrow from the number and general character of these cells. In recent years other features of young red cells have become of even greater usefulness, polychromatophilia and reticulation being of most importance. The reason the nucleated red cells are not invariable indicators of marrow activity resides in the fact that extensive marrow hyperplasia may exist without their appearance in the circulation. When, however, normoblasts are found, no one fails to regard them as bringing valuable evidence of active marrow growth—conditions of marrow disintegration being excluded. On the other hand, the older, non-nucleated, polychromatophilic or reticulated cells enter the circulation more readily than normoblasts and thus become more sensitive indicators of the condition of the marrow.

There are two ways of inducing normal marrow proliferation: first, by blood loss, and second, by exposure to atmospheres poor in oxygen. The literature upon blood loss is vast but in relation to the problem of blood-cell delivery from the marrow it is necessary to comment upon but two aspects of the subject. If animals are bled repeatedly and in small amounts, although occasionally one may find normoblasts in the blood, it is possible, by keeping the blood withdrawal low, to induce in the course of several months a high degree of marrow hyperplasia without the appearance of any considerable number of nucleated red cells. If, however, the hemorrhages are large, normoblasts and even megaloblasts are found in the blood-stream. They appear transiently, sometimes in extraordinary numbers, and their appearance invariably precedes or accompanies a period of active increase in the number of red cells in the circulation. Their appearance thus indicates an active effort on the part of the marrow to keep pace with blood loss. It is noteworthy that these crises of normoblasts, so easily produced

in animals subjected to isolated large hemorrhages, do not take place immediately after the blood loss, provided one has permitted numerical regeneration from the preceding hemorrhage to occur, but appear after a latent period of some days. It is our belief that the course of events in such cases is as follows: The marrow at the moment of blood loss consists, as we have pointed out, of a mixture of elements, all of which are incompressible and some of which are comparatively immovable. Immediately following the reduction in blood-cells, well-nourished animals begin to form blood more actively than before. Division of blood-cells within the marrow requires more space or else abnormally young forms will be forced into the circulating blood. This space, up to a certain point, is provided by movement of water out of the loose marrow reticulum. The tissue simply becomes drier, the blood-cells more closely packed. As cell division goes on, a stage is reached in which something must be removed besides water, and the fat cells, so numerous in adult marrow, are the next to go. Their removal depends upon processes of absorption which are slow, and, as a consequence of this sluggishness of removal, a period of overcrowding exists during which the easiest relief offered is found in the yielding blood vessels. For a time, therefore, young marrow cells, particularly the red-cell elements which develop near the capillaries, grow through the capillary walls and are swept off by the blood current.

It may be contended that in such a process it is impossible to have normal fatty marrow with widely open blood-vessels in one portion of the marrow cavity, and intense hyperplasia with encroachment of the growing blood-cells upon the vascular lumina in another. A little consideration will show that this is not the case, and that growth may cause active delivery of cells in one part of the tissue, with little alteration in the appearance of other parts. This arises from the fact that the fat cells, being incompressible within the limits of pressure possibilities in the tissue, continue to occupy an unaltered amount of space, and where they are numerous tend to support not only one another but also the large and yielding vessels which run in the arched crevices between them. The cells forming blood lie, in their turn, between the fat cells and the capillary wall, and, until active multiplication of these elements begins, the typical appearance of hyperplastic marrow—irregular encroachment upon the capillary lumen—will not be noticed. It is true that we have shown an instance of a closed capillary (fig. 28) and that smoothly closed capillaries of similar appearance can be found in actively growing marrow, and, to a less extent, in

non-hyperplastic normal marrow. It is, of course, possible that such capillaries are in periods of tonic closure similar to those observed in other tissues, and it is also possible that, in certain areas, they may undergo slight alterations in form and in position owing to the growth pressure transmitted through the fat cells from somewhat distant areas of dividing blood-cells. This pressure results in a smooth closure of the capillary in question. It is not easy to see how the capillary pictured in figure 28 could expand so as to conduct blood in effective amounts without some shift, change in position, or absorption of the neighboring fat cells. It, therefore, seems to us that pressure capable of closing capillaries may be transmitted through and provided by the fat cells in regions where their geographical relations to the capillaries happen to make this possible, but that in other regions of fatty marrow—and this will be true for the greater part of the tissue-growth encroachment upon blood-vessels—such pressure will not develop until active multiplication of near-by red cells begins and continues to a point of actual crowding.

The abrupt or "critical" appearance of nucleated red cells following large hemorrhages has been pictured in detail (21) in a series of dogs followed over a number of months. These observations bring out the surprising fact that after several hemorrhages the animals carry out the restoration of blood with less and less loss of normoblasts into the circulation, and that, eventually, they are able to experience large hemorrhages with practically no loss of normoblasts whatsoever. The reason for this adaptation is apparent, and rests upon the fact that in adult animals the space available for blood formation inside the bones is larger than the animal can maintain fully filled with actively growing red cell forming tissue. We do not know what red cells and, particularly, hemoglobin are formed from, but it does seem apparent that there is no form of stimulation which will result in completely filling the available marrow space with red cell forming elements, leucocytes being still present in sufficient numbers to keep the blood composition normal. This being the case, it is obvious that after a time an animal bled repeatedly will reach a stage in which he possesses a far larger amount of red marrow than is normal, but, owing to fat and even to bony absorption, this tissue now occupies a space surrounded—if we may make our statement somewhat diagrammatic—by a sufficient amount of water so that slight accessions in the rate of blood formation, if they occur following hemorrhage, are cared for by water movement alone. Overcrowding does not occur any more

than it does immediately following a single hemorrhage in an animal not previously bled. The capillaries have become normal, intact structures and the conditions of blood formation are those observed during the quiet formative equilibrium characteristic of the unbled animal.

In view of such considerations, it is not remarkable that animals bled in small amounts and at repeated intervals fail to show nucleated red cells in the circulation. The reason for this resides in the fact that the stimulus for cell multiplication is never so intense as to cause this process to outstrip the removal of fat. Adjustment of space keeps pace with growth, and the composition of the circulating blood does not present significant abnormalities. The actual amount of red marrow is not important. It is the degree of crowding incident upon rapid growth which produces changes.

Similarly, one can easily understand why normoblasts have practically never been seen in the blood of animals subjected to low atmospheric oxygen (48) (49) (50) (51). Schauman and Rosenquist (52), in a series of experiments in which they reduced pressure suddenly, were able, in a few cases, to obtain evidence of sudden normoblast extrusion but never of large magnitude. Their observation in this regard is an isolated one—if we except the experiments of Gaule (53), who made observations upon blood films in two balloon ascensions. The elevation reached was not great—4200 meters at most—so that the immediate effect of oxygen lack could not have been intensely acute. Yet this author reports blood films loaded with normoblasts, and publishes photomicrographs to illustrate this fact. We are inclined with Zuntz (48) to regard the evidence presented by these photomicrographs as due to artefacts and question the results emphatically.

In this explanation of the appearance of normoblasts in the circulation following large hemorrhages in which a definite anemia has been produced, of the failure of these cells to appear in more chronic hemorrhage from blood loss carefully adjusted so that practically no anemia nor blood dilution occurs, and of their failure to appear in the case of exposure to oxygen-poor atmospheres, where again no blood dilution takes place, our reasoning has concerned itself with the offering of immature cells to the circulating blood. We have assumed that, once placed in the path of a moving current of blood, normoblasts will be swept from the marrow. It will be noted, however, that the blood differs in these three sets of circumstances—in the first case, being usually somewhat dilute, freer of corpuscles per cubic millimeter, and

in the second two, very cellular. This difference in cellularity does not necessarily extend to the protein composition of the plasma, which, by the time normoblasts are ordinarily swept into the circulation, is well back to a normal level. In a large number of observations (21) it has been possible to show that bleeding and consequent blood dilution *per se* have, at the most, but slight *immediate* effect upon normoblast removal from the marrow. Removal occurs if the tissue is offering cells to the blood stream, and under no other circumstances, except during marrow disintegration. As a result, one must consider that the stimulus applied to the blood-forming tissue as a result of large hemorrhages is more intense than that applied by small hemorrhages or by atmospheres low in oxygen.

There is a peculiarity attendant upon this whole matter which merits comment. On passing from sea level to high altitude men often experience a series of unpleasant symptoms making up the "mountain sickness" syndrome. This state has been referred to oxygen lack. Loss of blood through hemorrhage may produce a few of the symptoms of mountain sickness, but there is no real similarity between the clinical states produced. One may suffer a hemorrhage severe enough to cause normoblasts to appear in the blood-stream, and yet have no symptoms aside from weakness, dyspnea on exertion, and pallor. On the other hand, rapid passage from sea level to an altitude of 14,000 feet may produce serious collapse, with headache, vomiting, etc., a syndrome of far greater severity. While it is true that, as yet, we have not sufficient knowledge to make fair comparisons between the physiology of hemorrhage and the adaptation to high altitude, it does seem that the former, if kept within reasonable limits, is capable of stimulating blood formation far more vigorously than the latter, though severer general symptoms attend the high altitude experience. It seems as though something entered the blood after blood loss, which is peculiarly potent in causing blood-cell development, and in the absence of this purely hypothetical substance in large amounts—the case in the high altitude experience—less vigorous blood-cell division occurs.

Evidence that growth within the marrow cavity will cause nucleated red cells to appear in the circulation is not confined to the red cells alone. Ewing (13), Rieder (14) and Sherrington (15) have noticed nucleated red cells in the circulation as a result of highly active leucocytic proliferation. Minot (54) has called attention to the frequent appearance of large numbers of normoblasts in the circulation under conditions of metastatic tumor growth in the marrow. In acute cases

of myelogenous leukemia in which myeloid tissue is rapidly filling the entire marrow cavity, normoblast extrusion occurs very prominently, and in this disease the finding of many such cells in the circulating blood is a bad, rather than a favorable sign.

There is a final aspect of marrow physiology which we have already discussed but which merits further elaboration. The very significant discovery by Doan (24) of large numbers of capillaries not transmitting blood has already been mentioned, and our own finding of closed capillaries of a somewhat different type has also been discussed. In muscle, Krogh (5) has shown that capillary closure and capillary opening bear a definite relation to the activity of the tissue, and Richards (55) has recently demonstrated an apparently similar possibility in the case of the kidney. In the perfused bone-marrow it has been possible to show (1) that vessels contract on stimulation of the nerve entering the marrow cavity with the nutrient artery, and following injections of adrenin. We have been able to repeat these observations in marrow circulated normally by the animal, and we have seen, furthermore, that on complete asphyxia of the perfused marrow tissue the rate of blood-flow may increase, a change due apparently to vascular dilatation. The reactions resulting in altered blood supply take place slowly in the marrow circulated with blood. It is clear that they cannot occur without a movement of water from the marrow parenchyma into the blood-vessels, or *vice versa*. Krogh (5) has made it quite clear that vasomotor reactions involve not only the arterioles but also the capillaries. It is not, however, possible to determine whether capillary dilatation or constriction occur as independent events in the marrow, and we, as yet, have no evidence that the first reaction of the marrow to stimuli bringing on intense activity of the tissue consists in an opening of closed capillaries—an increased vascularization of the blood-producing organ. At one time we believed that our injections of marrow in early stages of hyperplasia showed more capillaries per unit of area than could be found in normal marrow. We have not, however, been able to obtain assurance upon this important point and must await further work by Doan in order to settle the matter. We are, however, ourselves convinced, and feel sure that any one injecting marrow in advanced stages of hyperplasia will arrive at the same conclusion, that blood-cell growth may eventually entirely obliterate the capillary circulation in large areas of marrow. It is this reduction in blood supply which limits growth and causes extension through less encroached-upon regions of the marrow cavity.

With these facts in mind upon the growth-pressure delivery of red cells to the circulation and upon the general vascular arrangement and reactions of the marrow, let us see how the experiments described in this paper reach the issues involved. One may ask, first of all, why the elaborate procedure of marrow perfusion was necessary in order to develop the material we have been discussing. The first two perfusions upon dogs U. U. and N. N. (pp. 23 and 29) demonstrate that one may circulate the bone-marrow of dogs, which are, in so far as can be judged, in perfectly normal states of blood formation, at extraordinary rates of flow and high pressures without causing dislocation of nucleated red cells. Histological examination of the marrow tissue taken from these normal animals shows that the perfusing blood has been confined within definite capillary walls and does not flow directly over marrow cells, or through openings in the walls. These experiments furnish evidence that blood-cells are formed normally outside the blood-current, and that increases in blood movement alone will not dislocate marrow cells. Experimental evidence is thus offered that some other factor, inherent within the marrow, must be operative in order to cause red-cell movement from the marrow tissue to the circulation. In the case of the third perfusion, dog D. D. D. (p. 34), a puppy in a condition of intensely active blood formation was perfused at a high rate of blood-flow and a slight increase of pressure, and the manoeuvre resulted in a large dislocation of nucleated red cells from the bone-marrow. On microscopic examination, the marrow from this animal shows capillaries in which the endothelial lining can be seen only as a fragmentary structure or in which it is entirely absent. The circulating blood was thus in direct contact with the cell-bearing marrow stroma in many parts of the tissue, and was able to wash loose cells which had been brought into its path as a result of growth in the bony case of the marrow.

With these three experiments in which the actual process of cell delivery was followed during a definite period of time and under definite conditions, certain injection experiments may be correlated. The marrow of rabbit Yb (p. 56), a normal animal, injected under normal conditions of pressure, shows the injection mass distributed through large smooth-walled capillaries, many of which possess endothelial outlines. Figure 31 demonstrates this. In figures 32 and 33 the character of the capillaries in a more cellular region of typical normal rabbit marrow, taken from a non-hyperplastic animal, no. 3 (p. 61), are shown. Again the capillary outlines are regular and there has

been no escape of the injection mass into the marrow stroma. These animals are comparable to dogs U. U. and N. N. Rabbit Xa and cat Xd (pp. 62 and 67) represent animals injected at moments of active delivery of nucleated red cells from the marrow. They are comparable to dog D. D. D., and in figures 37, 38 and 41 one sees the irregular "moth-eaten" character of the margins of the injection mass and the impossibility of identifying any continuous endothelial wall.

This group of experiments, therefore, brings evidence that the marrow circulation is not an open one, that the circulating blood, unaided by alterations in the position of the marrow cells, cannot remove them from the marrow, and that, when conditions are favorable for the removal of nucleated red cells, marrow hyperplasia must be active. It thus becomes possible to hold that the stimulus for blood-cell growth is at the same time the stimulus for delivery of the cells to the circulation.

In order to gain additional evidence relative to the material just summarized, our experiments have dealt also with conditions in which disintegration of the marrow has been produced. The experiments of Bunting (30) furnish evidence that ricin and saponin cause breakdown of the marrow endothelium. This breakdown results in widespread exposure of marrow cells to the circulating blood and, as a result, immature red cells first, and later immature cells of other types appear in the blood in enormous numbers. The extreme conditions provided by saponin are reproduced less vividly by other substances. Timofejewsky (56) showed that normoblasts appear in the blood of dogs and rabbits very shortly after the injection of large doses of septic material. Such experiments as these, in which normoblasts appear almost immediately, are, we believe, merely expressions of the same sort of process that results from saponin. The effects of saponin have been described as being largely upon the marrow capillaries. Key (57) has recently called attention to another possibility and has made a valuable suggestion relative to the organization of the bone-marrow. He remarks:

> There is one very definite characteristic of the membrane of the young red cells which persists until after the reticular substance disappears from the cell. This is the stickiness of the outside of the membrane which causes reticulated cells to adhere to one another, to white cells, or to any object with which they are brought in contact, except mature erythrocytes. This tendency to agglutinate, or to adhere to other cells, offers a reasonable explanation of certain phenomena connected with the delivery of erythrocytes into the circulating blood. The

young cells simply stick together where they are formed until the cell membrane is matured (apparently by a hardening of its surface) when the cell is delivered into the circulation.

Key believes that this "stickiness" is the factor which results in the selective delivery of red cells to the blood, the young, stickier forms remaining together and the mature, non-sticky cells being washed away by the blood current. His evidence for stickiness in young forms consists in observations upon the tendency of reticulated cells to agglutinate. In this relation, it is interesting to note that one of the investigators concerned with this work of ours has often contended that nucleated red cells must be formed as a sort of syncytium, since in blood films one so frequently finds two or more close together and even at times directly in contact. Such a revolutionary observation is more probably an expression of the tendency of the young red cells to adhere to one another. In our opinion, Key's work forms a suggestive addition to our conception of marrow organization and should be subjected to further observation along the lines laid down by Fenn (58) in his studies upon hemolysis and stickiness in adult erythrocytes. One cannot examine the marrow after administration of saponin without feeling that some other factor in addition to capillary-wall destruction has been operative. The entire tissue seems loosened up. The different types of young blood-cells have lost all contact with their fellows and lie in isolation in the marrow. It therefore seems to us possible that agents causing the appearance of young blood-cells through direct attack upon the marrow do so both by capillary-wall destruction and by affecting the cohesion of the young marrow cells. Ether—indeed, anesthetics in general—foreign proteins, hirudin, hemolytic sera (59), all increase the number of nucleated red cells found in the blood of animals, and they do this most markedly in periods of active marrow growth when the young red cells directly border the bloodstream. In the experiments so far cited in which perfusion resulted in removal of nucleated red cells from the marrow, the hirudin added to the perfusing blood to prevent clotting, together with the prolonged anesthetization of the animal, may have produced conditions favorable to the dislocation of immature red cells by loosening the cohesion of these cells one to another.

Let us now see how our experiments upon marrow disintegration relate to the general problem of marrow organization and activity.

In three perfusions, dogs J. J., K. K., and H. H. (pp. 40, 45 and 49), the bone-marrow was circulated with blood completely saturated

with carbon monoxide. These animals were in different stages of marrow activity at the time of perfusion. The first, J. J., had been bled a number of times, but prior to perfusion had returned to a state of normal marrow organization. He simply possessed a larger amount of bone-marrow than the normal animal, but the relations between blood-vessels and marrow cells were not essentially different from the situation found in animals in quiet formative equilibrium. Perfusion under condition of complete asphyxia eventually resulted in marrow breakdown in this case, as evidenced by the appearance of nucleated red cells in the perfusing blood. In the two remaining perfusions, K. K. and H. H., perfusion was instituted in actively growing marrows and resulted in the delivery of normoblasts to the circulation. Figures 24 and 27 show the appearance of the marrow in these last two cases and it is evident that the perfusing blood has wandered into the marrow tissue. With these perfusions we may correlate certain injection experiments following saponin administration. The marrows of cat 12 C (p. 70) and of rabbit 8 (p. 71) were injected at a period of acute cell loss from the marrow. Figures 43 and 44 show that a completely open circulation existed and that in its presence the blood-forming tissue was simply swept into the blood-stream.

Our experiments upon marrow disintegration thus show the consequences of an open circulation in the marrow. This condition can exist for brief periods of time in hyperplastic bone-marrow tightly packed with blood-cells and, under such circumstances, the low pressure and sluggish flow of the blood in the marrow capillaries results in the dislocation of comparatively few cells. If, however, by the use of a substance like saponin, we entirely abolish the capillary outlines and reduce all parts of the marrow to the open-circulation condition, the tissue simply disintegrates—washes away in the circulating blood.

VI. CONCLUSIONS

By the use of methods of perfusion and of injection, so organized as to approach the physiological limits set by the animal under experiment, it has been shown that:

1. The capillaries conducting blood in the bone-marrow of the mammal in a condition of normal blood formation are closed structures, lined throughout with endothelium, and not in communication with the marrow parenchyma.

2. Under conditions of active red blood-cell formation, the extremely delicate walls of these capillaries are grown through by irregularly

placed red cells in varying stages of maturity. The capillaries are thus, for a period of varying length, open structures, but the opening presented does not result in flooding the marrow parenchyma with blood because of the packing of the immature blood-cells, which is an essential phase in the process of encroachment upon the capillary wall.

3. The normal mature erythrocytes are delivered to the blood-stream through the extraordinarily thin endothelial membrane lining the capillaries. This process must occur constantly and under the influence of such slight difference in pressure between the outside and inside of the blood-vessels as to cause no actual vascular rupture.

4. If further investigation should show that red cells develop intravascularly in endothelial bays temporarily out of the path of the blood-current, it should not alter the conception of cell delivery experimentally developed in this paper, since again the problem of accommodation of a growing tissue in a closed space must enter the physiological analysis of the problem.

5. The stimulus causing growth of red blood-forming tissue is responsible also for delivery of these cells to the circulation.

In conclusion, it is a pleasure to express our thanks to Dr. John A. Paul, Dr. Henry A. Kreutzmann and Mr. Charles A. Doan, who at varying intervals between the years 1915 and 1921 have assisted in the development of the experimental procedures we have described; to Dr. Florence R. Sabin, whose advice and interest have been a constant encouragement and help to us; and to Dr. William H. Howell, in whose laboratory and on whose suggestion these experiments were begun.

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MORPHINE HYPERGLYCEMIA AND THE ADRENALS[1]

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We have shown that the hyperglycemia associated with etherization, asphyxia (1), (2) and piqûre (3) is not essentially dependent upon the liberation of epinephrin from the adrenals, since it can be obtained, and apparently in as high a degree as in normal animals, when the adrenals have been removed or the epinephrin output from them interfered with.

We have now to report observations upon another form of experimental hyperglycemia, that produced by morphine, in which the adrenals appear to intervene in some way, at least to the extent that the occurrence of a definite hyperglycemia is far more constant when the adrenals have not been interfered with than after the various adrenal operations practised by us (removal of both adrenals in rabbits, removal of one adrenal with denervation of the other, or of one adrenal and a large portion of the other with denervation or destruction of the medulla of the remaining fragment in cats and dogs). For convenience we speak of the operations mentioned as "complete" adrenal operations, in contradistinction to those in which one adrenal only was removed and the innervation of the other remained intact. The results are summarized in tables 1 to 7, in which are displayed the initial rectal temperature before administration of morphine and the maximum (or minimum) temperature observed thereafter; the initial blood sugar content and the maximum content after morphine; the final blood sugar content and the effect produced upon it by a period of intermittent asphyxia; the percentage of glycogen in the liver at the end of the experiment; the body weight and the dose of morphine sulphate per kilogram of body weight. In tables 1 to 3 an additional column indicates the degree of severity of the general symptoms caused by morphine in the cats.

[1] A report of this work was made at the New Haven meeting of the Federation of American Societies for Experimental Biology, December, 1921.

Usually about six blood sugar determinations were made on each animal. The Folin-Wu method was used in the animals numbered 641 to 756 inclusive, and the Benedict-Lewis method (Pearce's autoclave modification) in those numbered 599 to 640. After the last blood specimen had been obtained the animal was killed by a blow on the head, the liver immediately excised and weighed and the glycogen in it estimated by Pflüger's method, the sugar being determined according to Bertrand. The rectal temperature, pulse rate, respiratory rate and the general behavior of the animal were noted from time to time.

Although we have sometimes thought that the morphine hyperglycemia did not depend so closely upon a good hepatic glycogen store as the other experimental hyperglycemias studied by us, we endeavored in nearly every case to favor the accumulation of glycogen by a suitable diet. For a few days after the final adrenal operation the cats and dogs received only milk. In the earlier experiments they were then fed regularly with as much as they could eat of a mixture consisting of 3 pounds of potatoes, 1 pound of rice, 2 pounds of liver, 1 pound of cane sugar, and 3 or 4 quarts of water, the whole boiled down to a stew. In addition they got milk 2 or 3 times a week. Every second day this diet was alternated with raw beef heart and occasionally fish.

Later it was found that the animals did better, as regards laying on glycogen, when they only received this special diet for a few days before the experiment, being kept the rest of the time on the ordinary diet, consisting of raw liver, beef heart, milk, and occasionally canned salmon, and once a week the stew described but without the sugar.

In a number of the animals the superior cervical ganglion had been excised on one side, and the changes in the pupil, nictitating membrane and the width of the aperture were compared with those in the normal eye. No essential difference was found between the animals whose adrenals had been interferred with and the controls as regards the reactions of the denervated eye.

In this connection it may be stated that incidentally both superior cervical ganglia were removed simultaneously from 4 cats and 5 rabbits. The ganglia were preserved for examination, and there is no reason to doubt that the extirpation was complete. The test which Meltzer (4) considered as indicating complete removal (dilatation of the pupil and retraction of the nictitating membrane after subcutaneous injection or instillation of adrenalin into the conjunctival sac) was positive in each case.

Of the 4 cats one died on the 44th day. The necropsy showed double pneumonia with pus in the chest. Another died after 3 months from unknown cause. The other 2 cats are alive 10 months, and 7 months after the operation, in good health and considerably increased in weight.

Of the 5 rabbits, one died on the 3rd day after the operation. The lower lobes on both sides were congested and consolidated in diffuse areas and the upper lobes congested. The remaining 4 rabbits are alive and well many weeks after the operation. It is not possible to say whether the pulmonary lesions seen in the rabbit which died were specially related to the operation. We lose a certain number of animals from pneumonia when no operation has been done in the neck e.g., after adrenalectomy, or without operation. The death of one of the operated cats more than 6 weeks after excision of the ganglia cannot be directly connected with that operation with any probability. So that out of 9 animals we have only seen one die under conditions which might suggest that the operation was responsible. We entirely agree with Meltzer (4) that it is not difficult to avoid such injury to the vagi as would cause death. In one experiment the superior cervical ganglion was excised on one side in a rabbit and the vagus divided on the other. The animal recovered perfectly and is now alive.

Pye-Smith (5), in the only instance in which he seems to have removed both ganglia from a rabbit, states that the animal survived, but was killed on the 12th day after excision of the second ganglion because of an abscess in the neck.

TABLE 1

Normal cats

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT	SYMPTOMS
	Before morphine	Maximum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia				
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*	
599	38.11	40.61	0.136*	0.184	0.184	0.238	0.18	13.0	2.38	Slight
603	38.50	42.50	0.156*	0.375			0.31	12.0	3.32	Marked
608	38.55	40.78	0.092*	0.184	0.096	0.142	3.11	3.3	3.06	Slight
609	38.28	41.11	0.130*	0.260	0.148	0.177	3.66	8.0	2.52	Moderate
610	37.44	41.67	0.142*	0.230	0.111	0.120	0.20	7.0	2.20	Moderate
613	39.50	40.55	0.086*	0.094	0.075	0.107	0.04	2.0	2.53	Slight
614	39.28	41.39	0.121*	0.220	0.132	0.204	2.50	2.5	3.95	Slight
618	38.39	41.72	0.107*	0.166	0.077	0.075	0.01	5.0†	2.97	Marked
664	38.86	41.70	0.080	0.267	0.093	0.216	1.85	12.1	2.87	Moderate
665	38.78	40.05	0.069	0.227	0.083	0.143	4.63	10.0	2.97	Moderate
666	38.63	41.80	0.069	0.266	0.111	0.182	0.30	12.5	1.95	Moderate
667	38.44	39.77	0.071	0.320	0.240	0.286	2.87	18.0	2.83	Slight
668	38.52	41.54	0.087	0.410	0.228	0.320	4.90	20.0	3.11	Moderate
669	39.30	41.60	0.100	0.238	0.082	0.170	0.01	3.6	2.75	Slight
670	39.15	39.82	0.069	0.100	0.072	0.220	0.88	1.9	2.61	Slight
705	39.45	41.45	0.083	0.182	0.083	0.357	1.39	14.0	1.82	Marked
708	39.50	42.12	0.070	0.093	0.070	0.100	Trace	13.0	1.73	Marked
746	38.77	41.61	0.074	0.200	0.102	0.263	2.59	13.5	2.75	Marked

* Blood sugar determination made by modification of the Lewis-Benedict method.

† Morphine given intravenously.

A specimen protocol of a typical experiment in a cat, a rabbit and a dog with the corresponding controls will serve to illustrate the course of the morphine hyperglycemia. In each case only four of the sugar determinations and two of the rectal temperature readings are included in tables 1 to 7. The changes in body temperature will not be considered further in this place, as the morphine hyperpyrexia in cats has

TABLE 2

Cats with miscellaneous operations

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT	SYMPTOMS
	Before morphine	Maximum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia				
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*	
652	38.90	41.88	0.063	0.200	0.105	0.190	0.28	13.5	2.99	Moderate
696	38.35	41.71	0.064	0.057	0.039	0.032	Trace	13.6	1.86	Moderate
697	39.60	40.71	0.097	0.091	0.060	0.060	0.47	13.0	2.31	Moderate
699	38.81	41.00	0.063	0.098	0.070	0.143	0.41	10.8	2.31	Slight
701	37.85	40.05	0.040	0.080	0.037	0.050	Trace	13.3	1.53	Slight
698	38.81	40.50	0.054	0.143	0.067	0.118	3.70	12.5	2.04	Moderate
659	38.50	42.00	0.091	0.133	0.100	0.204	4.79	9.5	3.16	Moderate
657	38.75	42.35	0.100	0.167	0.062	0.160	0.65	12.2	3.27	Marked
660	39.00	41.15	0.069	0.230	0.087	0.133	0.56	9.7	3.11	Marked
651	39.20	39.45	0.100	0.286	0.167	0.280	3.67	10.6	3.76	Marked
683	39.20	42.41	0.266	0.347	0.304	0.341	5.24	12.0	2.54	Marked
685	39.28	40.43	0.085	0.327	0.118	0.192	6.15	12.5	2.07	Marked

In cat 652 the right major, and the left major and minor splanchnics were cut. In 696 and 697 the splanchnics were cut on both sides, and the left coeliac ganglion with most of the right ganglion excised. In 699 and 701 the splanchnics were cut and the coeliac ganglia excised on both sides. The operations were done from 6 to 10 weeks before the experiment. In 698 the right splanchnic was cut. In 659 the left adrenal was denervated. In 657 the right adrenal was excised, and in 660 the left. In 651, 683 and 685 the spleen was excised.

been dealt with a previous communication (6). It may, however, be again pointed out that there is no necessary parallelism between the changes in blood sugar content and the variations in body temperature, nor is the hyperpyrexia affected by the adrenal operations in cats. Only in one cat (739, table 3) was there a fall of temperature after morphine, but this was very considerable, more than 4°C. This animal had a very high initial blood sugar content (0.25 per cent by

TABLE 3

Cats with complete adrenal operations

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT	SYMPTOMS
	Before morphine	Maximum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia				
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*	
611	37.83	40.83	0.120*	0.161	0.150	0.163	0.04	6.3	3.16	Moderate
612	37.78	41.94	0.133*	0.143	0.121	0.121	0.56	9.0	2.78	Slight
615	39.61	42.00	0.090*	0.120	0.101	0.092	0.02	9.0	2.70	Moderate
627	38.61	41.67	0.100*	0.102	0.086	0.115	2.50	12.7	2.33	Marked
639	39.00	40.05	0.092*	0.100	0.100	0.274	4.36	15.3	3.58	Slight
646	38.42	41.10	0.087	0.133	0.133	0.167	1.23	13.5	1.84	Moderate
647	38.50	40.15	0.067	0.105	0.105	0.235	0.77	12.0	2.47	Moderate
649	38.35	40.31	0.067	0.090	0.080	0.078	0.18	15.4	2.61	Marked
653	38.66	39.40	0.069	0.095	0.095	0.166	4.60	20.0	1.80	Moderate
654	38.68	41.69	0.067	0.166	0.091	0.083	5.01	25.0	3.12	Marked
656	39.20	41.11	0.080	0.133	0.100	0.220	0.56	12.5	3.22	Moderate
658	38.92	41.75	0.064	0.108	0.091	0.210	8.89	11.5	2.63	Marked
681	38.76	41.20	0.071	0.091	0.065	0.083	0.19	9.3	2.14	Marked
692	38.65	41.34	0.070	0.071	0.055	0.075	0.01	16.0	2.20	Moderate
693	38.50	40.56	0.060	0.089	0.074	0.129	0.02	11.4	2.21	Slight
695	38.78	40.91	0.067	0.093	0.080	0.100	Trace	17.4	2.28	Moderate
700	39.58	40.70	0.070	0.081	0.081	0.123	1.23	12.3	2.18	Marked
702	38.20	41.38	0.064	0.070	0.060	0.060	0.04	12.0	3.06	Slight
703	38.60	40.52	0.064	0.080	0.080	0.133	0.15	12.0	2.76	Slight
704	38.90	42.25	0.052	0.081	0.034	0.143	Trace	15.2	2.30	Marked
706	39.50	41.25	0.077	0.094	0.081	0.133	1.30	12.5	1.94	Moderate
707	38.12	42.66	0.062	0.064		0.210†	0.56	17.5	2.20	Marked
739	36.26	32.00‡	0.252	0.236	0.236	0.318	9.91	18.5	2.15	None
740	39.15	41.31	0.148	0.200	0.286	0.404	7.42	20.0	2.48	Marked
745	40.05	40.86	0.077	0.100	0.100	0.338	7.60	17.5	2.35	Slight
749	39.02	42.10	0.067	0.101	0.100	0.235	6.30	21.5	2.33	Marked
750	39.90	41.40	0.080	0.162	0.162	0.263	9.17	19.5	2.62	Marked
751	39.65	41.38	0.075	0.172	0.095	0.122	1.58	21.5	2.30	Marked

* Blood sugar determination made by modification of the Lewis-Benedict method.

† Animal died; heart blood obtained.

‡ Minimum temperature after morphine. The cat went to sleep under morphine.

TABLE 4

Control rabbits

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT
	Before morphine	Minimum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia			
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*
637	39.10	37.90	0.120*	0.260	0.151	0.214	3.34	21.7	2.29
641	40.00	38.72	0.092	0.223	0.150	0.220	4.17	27.0	2.82
643†	40.15	36.85	0.095	0.235	0.177	0.244	2.18	18.0	2.84
671	39.56	38.20	0.117	0.167	0.100	0.182	1.58	4.0	2.45
672	39.15	37.60	0.114	0.133	0.104	0.175	9.45	6.0	2.55
690	39.60	37.72	0.101	0.198	0.113	0.142	5.79	12.5	1.97

* Blood sugar determination made by modification of the Lewis-Benedict method.

† The kidneys were decapsulated, 10 weeks previously.

TABLE 5

Adrenalectomized rabbits

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT
	Before morphine	Minimum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia			
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*
630	39.44	39.00	0.083*	0.100	0.075	0.068	0.01	30.0	1.38
638	39.32	38.00	0.115*	0.115	0.094	0.194	1.32	25.0	1.98
640	39.44	38.80	0.110	0.106	0.085	0.100	0.62	21.7	2.32
642	39.00	37.30	0.125	0.111	0.093	0.153	3.37	28.0	2.70
644	39.00	37.40	0.090	0.143	0.114	0.108	0.17	18.0	2.82
650	39.15	36.90	0.118	0.117	0.105	0.200	4.90	18.5	2.71
662	38.60	37.11	0.108	0.108	0.085	0.135	1.83	14.0	2.47
663	39.03	38.10	0.100	0.133	0.105	0.190	5.47	12.0	2.45
684	38.91	41.13†	0.105	0.111	0.105	0.167	3.93	16.0	2.22
686	39.40	38.83	0.111	0.167	0.099	0.118	7.30	14.0	2.54
687	38.72	36.31	0.100	0.105	0.100	0.106	0.19	12.5	1.97
688	39.30	37.90	0.118	0.111	0.102	0.149	0.32	17.5	2.12

* Blood sugar determination made by the modified Lewis-Benedict method.

† In this animal the temperatue rose after morphine to a maximum of 41.13°.

Folin-Wu) and was obviously out of sorts, having suffered from diarrhea, possibly as a result of the special diet.

TABLE 6

Control dogs

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT
	Before morphine	Minimum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia			
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*
691	38.78	38.00	0.078	0.118	0.081	0.235	2.62	12.5	4.13
694	38.91	35.68	0.092	0.125	0.100	0.267	9.83	8.5	4.75
709	38.76	37.91	0.075	0.154	0.091	0.125	0.50	24.0	10.50
710	39.12	36.80	0.100	0.220	0.083	0.244	1.34	40.0	5.07
756	39.12	32.00	0.068	0.109	0.080	0.118	6.71	17.2	11.65
757	39.38	35.10	0.083	0.147	0.099	0.133	2.30	28.7	8.70

TABLE 7

Dogs with complete adrenal operations

NUMBER OF ANIMAL	RECTAL TEMPERATURE		BLOOD SUGAR				LIVER GLYCOGEN	MORPHINE PER KGM. BODY WEIGHT	BODY WEIGHT
	Before morphine	Minimum after morphine	Before morphine	Maximum after morphine	Before asphyxia	After asphyxia			
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*mgm.*	*kgm.*
661	38.86	33.64	0.091	0.113	0.077	0.148	6.90	10.0	5.19
689	39.26	36.90	0.081	0.085	0.080	0.147	2.75	9.3	7.41
711	39.10	37.56	0.074	0.083	0.074	0.156	0.46	60.0	4.22
721	39.70	37.38	0.080	0.083	0.083	0.091	7.40	50.0	4.50
722	38.98	36.15	0.083	0.105	0.105	0.192	3.95	32.0	7.90
723	38.69	37.70	0.082	0.085	0.081	0.133	12.60	35.0	6.70
731*	39.30	35.32	0.088	0.143	0.087	0.192	14.15	32.0	6.95

* In addition to usual operation, removed about ⅓ of remaining (denervated) gland.

In the rabbits the temperature fell after morphine, with one exception (an adrenalectomized rabbit, 684, table 5) in which there was a rise of more than 2°C.

In the dogs morphine caused invariably a fall of body temperature, which was usually considerable.

Condensed protocol. Cat 749; female; weight 2.33 kgm.

Right adrenal excised, left adrenal denervated, and left superior cervical ganglion excised, 14 weeks prior to date of experiment.

9:00 a.m. Temp. 39.02°; pulse 180; resp. 36; room temp. 24°C. Left pupil contracted and nictitating membrane forward.
9:03 a.m. First blood specimen contained 0.067 per cent dextrose.
9:05 a.m. Hypodermic injection of 50 mgm. morphine sulfate.
9:30 a.m. Getting active. Reflexes increased. Left pupil almost maximal, right about ⅔ dilated, both nictitating membranes slightly forward.
9:45 a.m. Temp. 39.46°; pulse 240 to 250; resp. 32. Active.
9:48 a.m. Second blood specimen contained 0.070 per cent dextrose.
10:35 a.m. Temp. 41.45°; pulse 260 to 280; resp. 44. Moderately active. Reflexes exaggerated. Eyes as above.
10:36 a.m. Third blood specimen contained 0.070 per cent dextrose.
11:45 a.m. Temp. 42.1°; pulse 240; resp. 56. Moderately active. Spastic. Eyes about the same. Tail hairs erect.
11:48 a.m. Fourth blood specimen contained 0.101 per cent dextrose.
1:00 p.m. Left pupil maximal, right wide but less than left, left nictitating membrane retracted, right slightly forward, left palpebral fissure wider than right.
1:20 a.m. Spastic jerks culminating in a short convulsion (lasting about 30 to 45 seconds). During convulsion the tail hairs were markedly erected.
2:05 p.m. Temp. 41.35°; pulse 280 to 300; resp. 48. Animal very spastic.
2:08 p.m. Fifth blood specimen contained 0.10 per cent dextrose. Now induced asphyxia off and on for 15 minutes. Usual vagus effect on heart observed.
2:30 p.m. Sixth blood specimen contained 0.235 per cent dextrose. Liver weighed 85.2 grams and contained 6.30 per cent glycogen.

Condensed protocol. Cat 705; male; weight 1.82 kgm.

Left superior cervical ganglion excised 7 weeks prior to date of experiment.

9:18 a.m. Temp. 39.45°; pulse 168; resp. 42; room temp. 22° C. Left pupil contracted and nictitating membrane forward.
9:20 a.m. First blood specimen contained 0.083 per cent dextrose.
9:25 a.m. Hypodermic injection of 25 mgm. morphine sulphate.
10:15 a.m. Getting uneasy. Left pupil maximal, right ¾ dilated, both nictitating membranes slightly forward.
10:55 a.m. Temp. 40.66°; pulse 152; resp. 48; moderately active. Eyes about the same as at last observation.
11:00 a.m. Second blood specimen contained 0.105 per cent dextrose.
12:15 p.m. Quite active. Eyes same as before. Pads moist. Tail hairs erect.
12:20 p.m. Temp. 41.22°; pulse 160; resp. 72.
12:25 p.m. Third blood specimen contained 0.182 per cent dextrose.
2:40 p.m. Temp. 41.45°; pulse 156; resp. 78; condition unchanged.
2:43 p.m. Fourth blood specimen contained 0.094 per cent dextrose.
3:10 p.m. Getting less active. Eyes same as at last observation.
3:53 p.m. Temp. 41.25°; pulse 180; resp. 40. Less active. Eyes about the same as at last observation.

3:55 p.m. Fifth blood specimen contained 0.083 per cent dextrose. Now induced asphyxia off and on for 15 minutes. Usual vagus effect on heart observed.

4:15 p.m. Sixth blood specimen contained 0.357 per cent dextrose. Liver weighed 62.2 grams and contained 1.39 per cent glycogen.

Condensed protocol. Rabbit 662; female; weight 2.47 kgm.

Right adrenal excised 9 weeks, left excised 8 weeks prior to date of experiment.

9:15 a.m. Temp. 38.6°; pulse 240 to 250; resp. over 300; room temp. 23.5°C.
9:20 a.m. First blood specimen contained 0.108 per cent dextrose.
9:25 a.m. Hypodermic injection of 35 mgm. morphine sulfate.
10:20 a.m. Temp. 37.61°; pulse 250 to 260; resp. 36.
10:25 a.m. Second blood specimen contained 0.108 per cent dextrose.
11:35 a.m. Temp. 37.11°; pulse 240 to 250; resp. 32.
11:43 a.m. Third blood specimen contained 0.085 per cent dextrose.
1:30 p.m. Temp. 37.12°; pulse 200 to 250; resp. 36.
1:45 p.m. Fourth blood specimen contained 0.085 per cent dextrose. Asphyxia off and on for 15 minutes. Vagus effect on heart observed.
2:05 p.m. Fifth blood specimen contained 0.135 per cent dextrose. Liver weighed 58 grams and contained 1.83 per cent glycogen.

Condensed protocol. Rabbit 690; female; weight 1.97 kgm.

10:00 a.m. Temp. 39.6°; pulse 200; resp. 88; room temp. 22°C.
10:20 a.m. First blood specimen contained 0.101 per cent dextrose.
10:25 a.m. Hypodermic injection of 25 mgm. morphine sulphate.
11:40 a.m. Temp. 38.62°; pulse 120 (slightly irregular); resp. 32. Somnolent.
11:45 a.m. Second blood specimen contained 0.198 per cent dextrose.
2:10 p.m. Temp. 37.72°; pulse 180; resp. 48. Sleeps.
2:15 p.m. Third blood specimen contained 0.167 per cent dextrose.
3:25 p.m. Temp. 38.9°; pulse 280 to 300; resp. 48. Less sleepy.
3:30 p.m. Fourth blood specimen contained 0.113 per cent dextrose. Asphyxia. off and on for 15 minutes. Vagus effect on heart observed.
3:50 p.m. Fifth blood specimen contained 0.142 per cent dextrose. Liver weighed 81.1 grams and contained 6.25 per cent glycogen.

Condensed protocol. Dog 722; female; weight 7.9 kgm.

The right adrenal was excised 7 weeks, and the left adrenal denervated 4 weeks prior to date of experiment.

8:50 a.m. Temp. 38.98°; pulse 120; resp. 24; room temp. 23.5°C.
8:55 a.m. First blood specimen contained 0.083 per cent dextrose.
9:00 a.m. Hypodermic injection of 250 mgm. morphine sulphate.
9:10 a.m. Getting sleepy.
9:50 a.m. Sleeps but is easily aroused.
10:00 a.m. Temp. 37.32°; pulse 68; resp. 30. Defecated.
10:05 a.m. Second blood specimen contained 0.080 per cent dextrose.
10:30 a.m. Dazed but wakeful and jerky (muscular twitching).
11:15 a.m. Temp. 36.42°; pulse 72; resp. 30. Condition the same.
11:18 a.m. Third blood specimen contained 0.086 per cent dextrose.
12:55 p.m. Temp. 36.15°; pulse 56; resp. 24. More wakeful and jerky.
1:00 p.m. Fourth blood specimen contained 0.093 per cent dextrose.
1:30 p.m. Getting less jerky and sleeps more.

3:00 p.m. Sleeps but is easily aroused.
4:30 p.m. Temp. 37.4°; pulse 70; resp. 24. Condition about the same.
4:40 p.m. Fifth blood specimen contained 0.105 per cent dextrose. Asphyxia off and on for 20 minutes. Usual vagus effect obtained with each period of asphyxia.
5:05 p.m. Sixth blood specimen contained 0.192 per cent dextrose. Liver weighed 360 grams and contained 3.95 per cent glycogen.

Condensed protocol. Dog 710; female; weight 5.07 kgm.

9:30 a.m. Temp. 39.12°; pulse 120; resp. 44, room temp. 21°C.
9:33 a.m. First blood specimen contained 0.100 per cent dextrose.
9:35 a.m. Hypodermic injection of 200 mgm. morphine sulphate.
9:50 a.m. Getting sleepy. Lying down.
10:00 a.m. Sleeping soundly.
10:55 a.m. Sleeps but is easily aroused. Temp. 38.15°; pulse 64; resp. 24.
11:00 a.m. Second blood specimen contained 0.220 per cent dextrose.
12:50 p.m. Temp. 37.98°; pulse 56 (irregular); resp. 22. Condition the same.
1:00 p.m. Third blood specimen contained 0.138 per cent dextrose.
2:45 p.m. Temp. 36.8°; pulse 60 (irregular); resp. 22. Condition the same.
2:50 p.m. Fourth blood specimen contained 0.097 per cent dextrose.
4:25 p.m. Temp. 37.0°; pulse 54 (irregular); resp. 20. Somnolent.
4:30 p.m. Fifth blood specimen contained 0.083 per cent dextrose. Asphyxia off and on for 15 minutes. With beginning of asphyxia the heart became, at first, faster and more regular, then became slow and regular; at end of each asphyxial period it returned to about the same condition as before asphyxia.
4:50 p.m. Sixth blood specimen contained 0.244 per cent dextrose. Liver weighed 217 grams and contained 1.34 per cent glycogen.

Comparing the normal and operated cats in tables 1 to 3,[2] we have 23 animals in which a complete adrenal operation was done and the blood sugar estimated by the Folin-Wu method. For controls with the same blood sugar method there are 10 cats in which no operation was performed, three in which a dummy operation (splenectomy) was done, two in which one adrenal was excised without interference with the other, and two in which one adrenal was partially or completely denervated. The 5 cats in which both splanchnics were divided, and in

[2] In cats 611, 612, 615, 627, 646, 703 and 745 one adrenal and a large part of the other were excised. In cats 639, 649, 695, 702, 704 and 706 one adrenal and a large part of the other were excised and the remnant denervated. In cats 647, 653, 654, 656, 658, 681, 692, 693, 707, 739 and 750 one adrenal was removed and the nerves, including the major and minor splanchnics, of the other cut. In cat 749 one adrenal was removed and the nerves of the other cut near the gland without division of the splanchnic. In cats 700 and 740 one adrenal was excised, the medulla of the other curetted out and the remnant denervated. In cat 751 one adrenal was excised, the medulla of the other destroyed with a drill, and a portion of the remnant tied off.

which therefore the innervation of both glands was interfered with (without removal of any adrenal tissue), may be omitted for this purpose. But their inclusion would cause no essential change in the result.

The average initial blood sugar content, that is, before morphine was given, for the 23 cats subjected to a complete adrenal operation (table 3) is 0.081 per cent. The average for 17 control cats (tables 1 and 2) is 0.090 per cent. One cat in each series (739 and 683) had a marked hyperglycemia before morphine was given (0.25 and 0.26 per cent of sugar). We do not think that this has anything to do with the so-called emotional hyperglycemia of certain writers, but that it may perhaps be explained as an alimentary hyperglycemia associated with the diet given the animals in order to fill up the glycogen depots. Certainly the initial hyperglycemia could not have been associated with an increased epinephrin liberation due to emotional disturbances, since it was seen in cat 739, in which the right adrenal had been excised and the left denervated. The protocol of this cat follows.

Condensed protocol. Cat 739; female; weight 2.15 kgm.

Right adrenal excised and left denervated, left superior cervical ganglion excised, 11 weeks prior to date of experiment.

9:35 a.m. Temp. 36.26°; pulse 216; resp. 24; room temp. 21.5°C. Left pupil somewhat wider than right; left nictitating membrane forward.

9:38 a.m. First blood specimen contained 0.252 per cent dextrose. The blood was dark colored and the flow very slow.

9:40 a.m. Hypodermic injection of 40 mgm. morphine sulphate.

9:50 a.m. Lying quietly in cage as if asleep.

10:15 a.m. Temp. 33.5°; pulse over 200 and weak; resp. 20. Reflexes slightly increased, but cat lies quietly in cage. Left pupil wide, right somewhat smaller, left nictitating membrane slightly forward.

10:20 a.m. Second blood specimen contained 0.220 per cent dextrose.

11:00 a.m. Temp. 34.2°; pulse 160; resp. 20. Condition about the same except that reflexes are now not exaggerated.

11:05 a.m. Third blood specimen contained 0.236 per cent dextrose.

12:20 p.m. Temp. 32.0°; pulse 70 to 76 (very weak); resp. 44. Animal appears to be very weak.

12:25 p.m. Fourth blood specimen contained 0.308 per cent dextrose. The blood was very dark and the flow slow. Just after this specimen was obtained the cat gave a few gasps and died.

Liver weighed 78 grams and contained 9.91 per cent glycogen.

The diet for this cat and two others included large amounts of cane sugar during a few days prior to the experiment. One of these cats died and the other (740, table 3) had a blood sugar content of 0.15 per cent before the morphine was administered, and 0.20 per cent and 0.29 per cent after morphine; then it died in a convulsion and the heart blood contained 0.40 per cent of dextrose. All three of these cats had profuse diarrhea.

If we omit the two animals (739 and 683) with marked initial hyperglycemia, the average blood sugar comes out 0.073 per cent for 22 cats after a complete adrenal operation and 0.079 per cent for 16 controls. For 12 control cats (tables 1 and 2) in which there was no interference with the adrenals or their nerves (omitting cat 683 with a marked initial hyperglycemia) the average blood sugar percentage was 0.075. It seems clear then that in cats after recovery from the adrenal operations the blood sugar content is within the normal limits.

The number of cats in which the sugar was estimated by the Benedict-Lewis method is too small to permit as close an agreement. The average for 5 cats after complete adrenal operations is 0.106 and for 8 normal cats 0.121 per cent. The blood sugar values given by this method in cats are materially higher than those yielded by the Folin-Wu method.

For the rabbits we did not find much difference, so that there can be no great harm in including the few Benedict-Lewis determinations (in three adrenalectomized rabbits and one normal animal) with those made according to Folin and Wu. The average initial blood sugar percentage for 6 control rabbits (table 4) was 0.106 and for 12 adrenalectomized rabbits (table 5) 0.106, precisely the same.

The estimations on dogs were all by the Folin-Wu method. The average for 6 control dogs (table 6) was 0.082 per cent, and for 7 dogs subjected to a complete adrenal operation (table 7) 0.083 per cent, before morphine was given.

It follows that in the cats, rabbits and dogs the hypoglycemia described by some writers after removal of the adrenals, and generally attributed to suppression of the epinephrin output, does not exist, at least when the animals have recovered from the operation.

It is true that the lowest initial blood sugar content seen in a cat (0.04 per cent) was in an animal (701) in which the splanchnics had been cut and the coeliac ganglion removed on both sides 45 days before the experiment. Here, of course, the adrenal innervation was extensively interfered with. But this was also true of the innervation of the liver. It is not known whether this circumstance, or the very small glycogen content of the liver (at the end of the experiment) was associated in any way with the low blood sugar content. An hour and a half after morphine was given, the blood sugar percentage was 0.08; an hour and three-quarters later, 0.06 per cent; and 2 hours thereafter, 0.037 per cent. After 15 minutes of intermittent asphyxia the sugar content was 0.05 per cent. During the asphyxia a good paradoxical

pupil reaction was obtained in the left eye, the left superior cervical ganglion having been removed at the time of section of the splanchnics.

When we compare the changes in the blood sugar content after morphine in the control cats with those in cats after a complete adrenal operation, two striking differences emerge. First, a hyperglycemia is far less common in the operated animals; second, when there is any increase in the blood sugar content, the maximum change is smaller and less rapidly reached than in the controls.

Thus, among the 28 cats in table 3 the blood sugar content was increased by 100 per cent or more after morphine in only 3 cases (cats 654, 750, 751). In none of these was the increase much above 100 per cent. In at least 12 cases there was either no change or the change was so slight as to fall within the limits of the normal variations.

In the 25 cats (tables 1 and 2) in which there was either no interference with the adrenals or their nerve supply, or interference with only one adrenal, the blood sugar content was increased by 100 per cent or more in 15. In many of these the increase was 200 to 300 per cent or more. In only 2 or 3 cases was the change so small as to render it doubtful whether a genuine increase had occurred or not.

In the rabbits the difference is fully as striking. In 4 out of 6 normal animals (table 4) the blood sugar was increased 100 per cent or more after morphine, in only one was the increase so small as probably to fall within the normal variations. In this case one of the smallest doses of morphine was given and the glycogen content of the liver was also the smallest in the series. In 12 adrenalectomized rabbits (table 5) only in 2 cases was the blood sugar increased by as much as 50 per cent after morphine. In 9 cases there was practically no change.

In 6 normal dogs (table 6) there was an increase of 100 per cent in the blood sugar after morphine in 2 cases; in the other 4 cases the increase was only 50 per cent or less. In the 7 dogs with a complete adrenal operation (table 7) the blood sugar remained practically unaltered after morphine in 5 cases. In one case there was an increase of about 50 per cent.

While the hyperglycemia caused in the control rabbits and especially in the control dogs was generally distinctly less pronounced than in the control cats, the adrenal operations seemed to interfere with the reaction in dogs and rabbits as in cats.

We have not been able to determine in what way the adrenals favor the production of morphine hyperglycemia. Since removal of one gland and denervation of the other (in cats and dogs) seems to interfere

with the reaction as effectively as removal of a greater part of the adrenal tissue, it might be supposed that it is the epinephrin output which is the important thing. We have shown (7) that in the cat morphine increases the epinephrin output, and it is clear that if the increase is sufficiently sustained, and great enough to bring the epinephrin content of the blood to the level at which hyperglycemia is produced when adrenalin is artificially injected, an epinephrin hyperglycemia must follow. There is this difficulty, however, that in the dog morphine causes either no increase in the epinephrin output or one which is insignificant compared with that in the cat. It is, of course, possible that the morphine hyperglycemia, without being an epinephrin hyperglycemia, is yet dependent upon the presence of a small amount of epinephrin. Be this as it may, it is differentiated from the hyperglycemia caused by etherization, asphyxia and piqûre by the fact that, as already mentioned, the latter forms are easily obtained after complete adrenal operations.

In the case of asphyxia this is illustrated again in the tables, since each experiment was usually completed by a period (generally 15 to 20 minutes) of intermittent asphyxia. This was done in order to see whether this form of hyperglycemia, one of the most easily obtained, could be elicited in each animal. The asphyxia hyperglycemia is closely dependent upon a sufficient glycogen store in the liver, and not at all closely dependent upon the presence of the adrenals. It will be seen that generally a good asphyxia hyperglycemia was obtained, when enough glycogen was present, in animals whose blood sugar content was unaffected by morphine. It must be remembered that the hepatic glycogen was determined at the end of the experiment, and it is impossible to know how much greater it might have been at the beginning, especially when a decided hyperglycemia had been produced and had continued for some hours. Some of the animals had only a small glycogen store, although fed with a diet supposed to be favorable to the accumulation of glycogen. The state of health of the animal, especially the condition of the digestive tract, its appetite, the weather, possibly the sexual cycle, are factors which may affect the glycogen store. It must be recognized also that, as regards the asphyxia hyperglycemia in these experiments, asphyxia was only induced after the animals had already been under morphine for a number of hours. Even when the blood sugar had returned to about its initial level before asphyxia was induced, in the cases in which a distinct hyperglycemia had been produced by the morphine, it is probable that the conditions

for eliciting a marked hyperglycemia by asphyxia were not as favorable as they would have been with a similar glycogen store, had no morphine been given. That the morphine hyperglycemia is not due to interference with respiration, as seems to be held by some writers, seems evident not only in the cat, in which the respiration is exaggerated, but also in the dog and rabbit, since in these animals after the adrenal operations morphine alone, even when it affected the respiration distinctly, did not generally cause hyperglycemia in anything like the same degree as asphyxia did. Also at a time when the hyperglycemia induced by morphine was disappearing, and the blood sugar might have returned to normal or become subnormal, the respiration was apt to be even more depressed than earlier in the morphine action when distinct hyperglycemia was present.

One other point ought to be made clear. In dogs, but especially in rabbits, with such doses of morphine as were employed, it was often impossible to asphyxiate the animal at the end of the experiment as thoroughly as could be done with the non-morphinized animal without causing death. Therefore in some cases the apparently small effect of asphyxia upon the blood sugar is probably due simply to the fact that the asphyxia was not sufficiently prolonged or sufficiently complete.

The fact that the asphyxia hyperglycemia is well obtained after complete adrenal operations proves that an increased epinephrin output is not an essential factor in producing it. Indeed there is no good evidence that asphyxia increases the epinephrin output. The statement of Gley and Quinquaud (8), repeated in a recent paper (9), that in asphyxia the rate of liberation of epinephrin is increased, is simply based upon confusion of the concentration of epinephrin in the adrenal vein blood with its rate of output per unit of time. They found that two or three times as much adrenal blood, collected from a dog without asphyxia, must be injected into another dog to cause a given rise of blood pressure as of adrenal blood collected during asphyxia lasting 2 to 4 minutes. All that this proves is that the concentration of epinephrin in the adrenal blood was increased during the period of asphyxia. In the absence of measurements of the rate of blood flow through the adrenals, with and without asphyxia, no deduction can be made as to changes in the rate of output. As a matter of fact, we have shown (10) that the average blood flow through the adrenals was diminished by asphyxia, for such periods as were used by Gley and Quinquaud, and that the concentration of epinephrin in specimens collected at different times during the asphyxial period was inversely proportional to the

blood flow, the rate of output remaining unchanged within the limits of error of the methods employed. This is in accord with the result of Gley and Quinquaud (8), (9), that the rise of blood pressure induced by asphyxia has precisely the same course and magnitude whether the adrenals are present or not. The statement of certain writers, that the chrome reaction is diminished by prolonged asphyxia if the nervous connections of the adrenal are intact, is compatible with a diminished formation as well as with an increased liberation of epinephrin. As a quantitative method, this reaction also leaves much to be desired.

It was not infrequently observed both in the normal cats and in cats whose adrenals had been interfered with, that the blood sugar content, several hours after the administration of morphine, might not only have come back to the initial level but might have dipped below it. This seemed sometimes, though not invariably, to be associated with a low hepatic glycogen content; occasionally it seemed to be associated with a rather poor condition of the animal.

Thus in a normal pregnant cat (618), the initial blood sugar (Benedict-Lewis method) was 0.107 per cent. Twenty minutes after the administration of morphine it was 0.158 per cent; an hour and a half later, 0.166 per cent. After a further interval of 2½ hours the percentage had fallen to 0.083; in an hour and a half more it was 0.077; after 25 minutes intermittent asphyxia it was unchanged (0.075 per cent). There was only a trace of glycogen in the liver.

In a cat (696), in which the splanchnics were divided on both sides and the whole of the left coeliac ganglion, with most of the right, extirpated, 75 days before the experiment, the initial blood sugar content (by the Folin-Wu method) was 0.064 per cent; 70 minutes after the giving of the morphine it was 0.062, and the same 90 minutes later. Four hours and 20 minutes after morphine it was 0.057, and 2 hours later only 0.034. Asphyxia for 15 minutes caused no increase (0.032 per cent). The liver contained only a faint trace of glycogen.

In another cat (697), after a similar operation, the initial blood sugar was 0.097 per cent (by Folin-Wu method). After morphine the readings were 0.074 (1 hour), 0.091 (2 hours), 0.091 (4 hours), 0.060 (6½ hours after morphine). Asphyxia for 15 minutes caused no change. The liver contained about 0.5 per cent of glycogen.

The degree of excitement of the cats and dogs before and during the withdrawal of the first specimen of blood was generally noted. Of the normal cats whose blood sugar was determined by the Folin-Wu method (including two from which the spleen had been removed 5 and 8 weeks prior to the experiment, and two in which the innervation of one adrenal only had been interfered with) five were classified as "quiet" and 9 as "excited." The sugar contents of the initial specimen for the quiet cats were 0.100, 0.080, 0.069, 0.085 and 0.074 per cent

(average 0.082). For the 9 excited normal cats the sugar contents of the initial specimen were 0.091, 0.069, 0.069, 0.071, 0.087, 0.100, 0.054, 0.083, 0.070 per cent (average 0.077).

For 10 cats with a complete adrenal operation, which are classified as "quiet" before and during withdrawal of the initial blood specimen, the sugar contents were 0.067, 0.069, 0.067, 0.064, 0.071, 0.064, 0.064, 0.052, 0.062, 0.067 per cent (average 0.065). For 15 cats with a complete adrenal operation (or with double splanchnotomy), which are classified as "excited" before and during withdrawal of the first blood specimen, the sugar percentages were 0.067, 0.063, 0.080, 0.070, 0.060, 0.067, 0.064, 0.097, 0.063, 0.070, 0.040, 0.077, 0.077, 0.080, 0.075 (average 0.070).

If the initial blood sugar contents of the 15 "quiet" cats, whether normal or operated, are averaged, we get 0.070 per cent, and for the 24 "excited" cats 0.072 per cent.

There is no evidence in these results on cats that the blood sugar content of the specimen taken before morphine was influenced by the degree of excitement of the animal. It must be remembered that the time needed to obtain a specimen of blood is short (a minute or two), and there would seem to be no possibility that any degree of excitement could so accelerate the velocity of a reaction like the hydrolysis of the hepatic glycogen as to sensibly alter the sugar content of the whole blood in so brief a time. We have previously published experiments (1) in which animals which had been particularly quiet during withdrawal of the first blood specimen were then purposely excited intensely for 20 minutes to an hour, without effect upon the blood sugar content beyond the ordinary variations in successive samples. It may be again pointed out that in attempting to demonstrate the so-called "Fesselungs" glycosuria or hyperglycemia, some investigators have kept the animals tied down for several or even many hours. Here the conditions are quite different, and other factors than emotional excitement may be involved. However this may be, we do not think that any real proof has ever been given that emotional disturbance causes an increased output of epinephrin, on which the alleged emotional hyperglycemia has often been supposed to depend.

The condition of the dogs as regards excitement was also noted, but the number of animals is too small to yield a satisfactory average. Two of the control dogs are described as "quiet" and three as "excited" during withdawal of the initial blood specimen. The sugar percentages for the quiet dogs were 0.075 and 0.068 (average 0.071), and for the

excited dogs 0.078, 0.092, 0.100 (average 0.090). For two dogs which had undergone a complete adrenal operation and which were described as quiet, the sugar percentages were 0.074 and 0.083 (average 0.078), and for five excited dogs, 0.091, 0.081, 0.080, 0.082, 0.088 (average 0.084). Putting the four quiet dogs (whether operated on or not) together, we get an average sugar content of 0.075 per cent. Doing the same for the eight excited dogs, the average comes out 0.086 per cent.

Since some writers are inclined to attribute to the adrenals, sometimes in conjunction with the thyroid, an important rôle in the regulation of body temperature, it may not be without interest to state that no influence of the adrenal operations on the rectal temperature of the animals can be detected by examination of the tables.

For 18 normal cats (table 1) the average initial rectal temperature was 38.75°C.; for 12 cats with miscellaneous operations (table 2) 38.85°C.; and for 28 cats with complete adrenal operations (table 3) 38.76°C. For 6 normal rabbits (table 4) the average temperature was 39.59°C., and for 12 adrenalectomized rabbits (table 5) 39.11°C. For 6 normal dogs (table 6) the average was 39.01°C., and for 7 dogs with the adrenal operations (table 7) 39.13°C. The agreement is excellent in the case of the cats, a sufficient number of animals being available for deducing a satisfactory average. The averages are also practically identical in the control and operated dogs. The somewhat greater difference in the case of the rabbits is almost certainly due to the small number of control animals, although possibly the fact that the observation on the rabbits were made mainly in winter may have had slight influence, the hair on the shaved surface in the operated animals being generally still rather short at the time of the experiment.

SUMMARY

1. Hyperglycemia is much less commonly induced by morphine in cats which have recovered after removal of one adrenal and denervation of the other, or after removal of one adrenal and a large portion of the other, with or without denervation of the remnant or destruction of the medulla of the remnant, than in normal cats or in cats which have recovered from such operations as splenectomy.

2. When morphine causes hyperglycemia in cats after such adrenal operations, the maximum increase in the blood sugar percentage is on the average much less than in normal cats, and is more slowly reached.

3. Dogs, after removal of one adrenal and denervation of the other, behave in the same way as cats, when compared with normal dogs in

regard to the frequency with which morphine hyperglycemia may be elicited, and the magnitude and rapidity of development of the hyperglycemia in the rather rare cases in which the blood sugar is distinctly increased. However, in our experience the morphine hyperglycemia, even in normal dogs, is usually less pronounced than in cats.

4. In rabbits which have survived double adrenalectomy and are in good health, the same difference (in the frequency and degree of the hyperglycemia induced by morphine) between the adrenalectomized and the normal animals is seen as exists between cats subjected to the adrenal operations described and normal cats.

5. An explanation of the phenomenon is not attempted at present, especially in view of the fact that the general behavior of cats under the influence of morphine is so different from that of rabbits and dogs. It does not seem probable that the morphine hyperglycemia in the normal animals is an "adrenalin hyperglycemia," due to the stimulating effect of the drug upon the epinephrin output, although we have shown that morphine increases the output in cats, since in dogs little if any increase in the epinephrin output has been demonstrated. It is nevertheless possible that although the morphine hyperglycemia may not be an "adrenalin-hyperglycemia," in the sense that the epinephrin content of the blood is raised to, and maintained at or above the threshold value necessary for the production of hyperglycemia when adrenalin is artificially injected, it may be facilitated by the ordinary output of epinephrin.

However this may be, the morphine reaction seems to be a test which distinguishes animals subjected to the various adrenal operations from normal animals, from which they may otherwise be indistinguishable. A knowledge of the mechanism of the morphine hyperglycemia may therefore throw light upon the physiology of the adrenals.

6. It is again shown that the asphyxia hyperglycemia, as was previously demonstrated for that form of hyperglycemia, as well as for that due to etherization and to piqûre, is in a high degree independent of the adrenals. The morphine hyperglycemia in this regard is therefore sharply differentiated from the other forms of experimental hyperglycemia mentioned.

7. The average blood sugar content, before morphine was given, of the cats, dogs, and rabbits subjected to the adrenal operations described was the same as the average for the corresponding groups of control animals.

8. There was no evidence that the degree of excitement of the animals, whether normal or after the adrenal operations, influenced the sugar content of the blood specimens collected before morphine was given. The average sugar percentage for "quiet," was approximately the same as for "excited" animals of the same group.

9. The average of the rectal temperatures of the control animals (before morphine was given) was the same as that of the animals which had undergone the adrenal operations.

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THE EFFECT OF ETHER ANESTHESIA ON AFFERENT PATHS IN THE DECEREBRATE ANIMAL

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As a result of certain histological observations, Crile and Lower (1) have concluded that "ether anesthesia offers no protection to the brain cells against the effect of trauma, and that the lipoid solvent anesthetics probably break the arc which maintains consciousness beyond the brain cells somewhere in the efferent path." They state that "the afferent path from the seat of injury being unbroken, the afferent stimuli reach and modify the brain cells as readily as if no anesthetic had been given." Many investigators have been impressed with the difficulties of drawing valid conclusions from histological examination of nerve cells, principally for the reason that the variations between individual cells in a given microscopic field are usually so great that it is almost impossible to make a fair selection of a typical cell. Some years ago Dr. W. B. Cannon suggested that we might obtain evidence bearing on this problem by connecting a string galvanometer with the brain stem of a decerebrate animal and noting the effect of ether anesthesia upon the electrical disturbance set up in response to afferent stimulation. A preliminary report on our earlier experiments has already been published (2). Since the publication of that report we have extended our series of experiments, and by the aid of certain modifications and improvements have elucidated some of the points which were still in doubt at that time. In the present paper, therefore, we shall attempt to make a more complete statement of our results and conclusions.

As indicated in our previous communication, the decerebrate animal constitutes a convenient preparation for examining the activity occurring in the first two neurones of the afferent chain from the periphery to the brain. The afferent path for the impulses of touch, temperature and pain, as far as it remains intact in the decerebrate preparation, consists in peripheral neurones terminating in the gray matter of the spinal cord, and ascending neurones passing thence by way of the medial

fillet to the thalamus. The path for conduction of the muscle sense consists in peripheral neurones passing forward within the spinal cord as far as the medulla oblongata and there ending within the nuclei gracilis and cuneatus, and ascending neurones arising at this point and passing forward as medial fillet to the thalamus. Both tracts are interrupted by decerebration in the course of the second neurones; and in the case of both tracts these second neurones lie near the surface in the medial fillet. Electrodes may, therefore, be applied within the cranium to points on the brain stem in the vicinity of portions of the second neurone in each type of conduction path. By connecting these electrodes with a string galvanometer we should expect to obtain evidence of action currents resulting from peripheral stimulation. Any peripheral stimulus sufficient to provoke sensaton in the intact animal would presumably cause afferent impulses in the fillet which could be detected in this way. The classical work of Gotch and Horsley (3) illustrated the possibility of detecting activity in various parts of the nervous system by means of action currents, but the lack of sensitive and rapid recording apparatus when their experiments were performed rendered impossible the analysis of observed activity into individual volleys of nerve impulses, which methods now available have brought within our reach.

The decerebrate animal, being devoid of consciousness and spontaneous movement, may conveniently be used to compare the central effects of intense peripheral stimulation with and without ether anesthesia. Our problem, therefore, was to apply stimulating electrodes to a large peripheral nerve and record with a string galvanometer the disturbance set up in the neurones of the fillet, while the animal was free from ether anesthesia; then to determine whether the response so obtained was modified by such ether anesthesia as is used in surgery, and if so, the nature of the modification.

The use of the action current as a criterion for the existence of a nerve impulse or rather the disappearance of the action current as proof of its cessation, might be criticized on the ground that the electrical disturbance has not been proved beyond doubt to be the inseparable concomitant of the nerve impulse (4). All the evidence at hand, however, supports the view that the electrical response is an essential element of the nerve impulse, without which conduction cannot occur (5). But in order to control this aspect of the problem, some experiments were performed to answer the specific question whether profound ether anesthesia could abolish the electric response in a nerve trunk,

without stopping the passage of the nerve impulse, as determined by a functional test. The results of these experiments have been previously reported (6), and they seem to show clearly that general anesthesia in a mammal, even when carried to the point of stopping respiration, does not materially impair the action currents which may be obtained from a nerve trunk upon direct stimulation; they further show that the direct application of ether vapor to a motor nerve trunk never causes the disappearance of the action current before the cessation of contraction in the innervated muscle. We may, therefore, conclude that the action current is a safe criterion of function for the purposes of our problem.

Method. The procedure in general was as follows. All experiments were performed on cats. Under deep ether anesthesia the carotid arteries were ligated, a tracheal cannula inserted, and the animal decerebrated with the Sherrington decerebrator or guillotine (7), the transection of the brain stem being made just in front of the anterior colliculi. Enough of the cranium was then removed to admit free access to the exposed brain stem. This was then covered with cotton moistened in warm Ringer's solution until all was ready to proceed with the observations. The head was raised slightly above the table and secured firmly in a stand which prevented such movement as might cause shifting of electrode contact on the brain stem. An incision was made in the thigh and the sciatic nerve exposed for 3 or 4 cm., ligated and severed peripheral to the ligature. The stimulating electrodes were applied to it, shielded in glass tubes by Sherrington's method (8), and the wound was sewed around the neck of the glass shield. For stimulation single break shocks were delivered by a Berne inductorium calibrated in accordance with Martin's method (9). In all the earlier experiments a signal magnet in the primary circuit was arranged to throw a shadow on the film (cf. 10). This showed the time of stimulation to within about 0.5σ.

The leading-off electrodes consisted of tubes containing a jelly of agar agar and Ringer's solution in the bottom of which was imbedded a bit of twine protruding an inch or so from the lower end. This twine, soaked in Ringer's solution, was applied to the desired point on the brain stem. Above the jelly in the tube was zinc sulphate and into this dipped an amalgamated zinc rod. In most of the experiments the string galvanometer was of the Cambridge type; in the last two it was of the Hindle type, a special instrument with an air gap of 1.5 mm. For recording galvanometric deflections we first used a camera which has been previously described (10). In the later experiments we used

a different camera adapted to operate with motion picture film, also described in a previous communication (11).

The preparation was usually ready for the commencemet of observations about an hour after decerebration. The administration of ether having ceased immediately after decerebration, there was very little of the drug left in the system at the expiration of this interval.

After recording a series of responses while the animal was thus free from the effects of the anesthetic, ether was administered by connecting with the tracheal cannula through a short rubber tube an ether bottle containing liquid ether and absorbent cotton. As the etherization progressed records of responses to maximal stimuli were taken at frequent intervals. At each stimulus the leg in which the nerve was stimulated was watched, and the presence or absence of flexion reflex was noted. The time of disappearance of the corneal reflex and, in most cases, of the pinna reflex (retraction of pinna when pinched with forceps) was also noted. Ether was administered till absence of reflexes indicated a deep surgical anesthesia, sometimes till respiration ceased. The ether was then withdrawn and, when necessary, artificial respiration was given. During recovery from ether records were taken at intervals until the recovery was complete, the return of reflexes being at the same time noted. In this way the progressive changes in the brain-stem responses were correlated with the depth of anesthesia as shown by the various reflexes, both in going under ether and in coming out.

Results. We found that it was usually easy to obtain evidence of electrical disturbance in the brain stem in response to maximal stimuli applied to the sciatic nerve. At first we applied one leading-off electrode, designated the active electrode, on the lateral surface of the brain stem in the region where the fillet comes nearest the surface, on the opposite side from the sciatic nerve to which the stimuli were applied. The other electrode, or inactive lead, was applied to the cut surface on the other side. In one experiment an exploration was made of the topographical distribution of the electrical disturbances. Stimulating electrodes were applied to both sciatic nerves, so that the stimulus could be applied to either at will. With the leads placed as described above, little or no reflection resulted from stimulation of either sciatic nerve. But when the leads were placed in the median plane, one at the top of the brain stem and the other at the bottom, considerable deflections were obtained on stimulation of either nerve, there being very little difference between the two. (See fig. 1, A and B.) When one lead was applied

to the posterior colliculus on the same side as the stimulated nerve and the other lead diagonally opposite to it at the ventral edge of the cut surface of the brain stem about 5 mm. from the median plane, we obtained the largest excursions of all (fig. 1, C). When the stimulus was applied to the opposite nerve, the leads being left at the same points, the excursion became much smaller (fig. 1, D). The direction of the excursion was such as to indicate negative potential in the electrode applied at the ventral edge of the cut brain stem. It is well known that

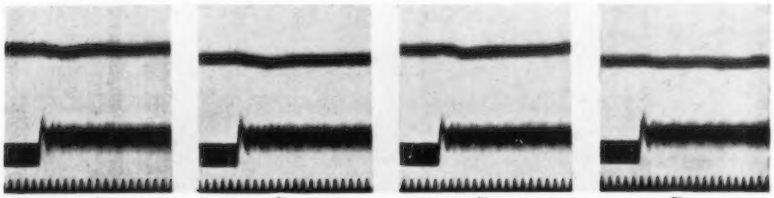

Fig. 1. Galvanometer records of electrical disturbance in brain stem with different arrangements of leading-off electrodes. Experiment 10. *A* and *B*, leads in median plane; "active" lead[1] dorsal, "inactive" lead ventral. *C* and D, "active" lead on right posterior colliculus, "inactive" lead on left ventral edge of cut surface. *A* and *C*, stimulus applied to right sciatic nerve; *B* and *D*, stimulus to left sciatic nerve. In every case stimulus was single break shock, 129 Z units.[2]

Cambridge galvanometer; "String C," a platinum string of 3μ diameter and 5000 ohms resistance. Magnification 580 diameters. Tension of string, 1 cm. excursion = 6.2×10^{-8} amp.

The upper line shows the excursions of the string; the second line is the shadow of the signal magnet; the lowest line records time, each complete vibration = 0.01 second.

a fall of potential indicates proximity to the active regions in an excited tissue. The observation, therefore, that the point of maximum fall of potential is to be found in the opposite side of the brain stem from the stimulated nerve is in accordance with what one would expect on anatomical grounds, although the finding of this point on the ventral

[1] The use of the term "active lead" is purely arbitrary; it means that when this electrode is at the point of activity (lowered potential) the excursion of the string in the galvanometer is such as to appear as a rise in the line as reproduced in the figures.

[2] With electrodes applied as in the experiments, to the sciatic nerve of the cat, it has been previously shown (Forbes and Gregg: This Journal, 1915, xxxix, 181) that break shocks of more than 50 Z units are usually maximal; i.e., apparently suffice to excite all the fibers.

rather than on the lateral surface was somewhat surprising, and its meaning is not altogether clear. Thereafter we adopted this diagonal arrangement of the leads as the standard in all experiments dealing with the brain stem (fig. 2).

The possibility that the galvanometric excursions might be due to shift of electrode contact resulting from reflex muscular contractions was ruled out by the following facts: The only reflexes evoked by the stimuli were the flexion reflex and occasionally the crossed extension reflex; observation showed that these caused no visible shift of the electrode contacts; when any shift occurred this was due to respiratory movements, and even when visible, this did not cause deflections in the galvanometer comparable with those following stimulation. The approximate uniformity of the form of electric response in a long series of experiments is incompatible with a fortuitous cause such as disturbance of contact. Finally, the latency of the electric response is too short to be explained as a result of muscular contraction. The flexion reflex is the quickest of the possible disturbing reflexes; in this case the electric response of the muscle has a latency of 10 or 11σ ((10), figs. 11 and 12); the mechanical response could not attain a magnitude sufficient to impart its motion to the lead in less than 15σ after the electrical response has appeared in the muscle. Therefore, the earliest possible mechanical disturbance due to the reflex would occur at least 25σ after the stimulus. In a later section it will be seen that in our experiments the first excursion of the galvanometer occurred about 8σ after the stimulus, and the point of maximum excursion had been reached in 26σ. All these considerations make it clear that the galvanometer was registering a true physiological response in the nervous tissue.

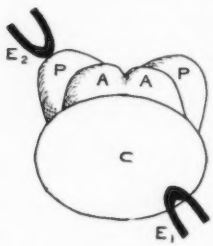

Fig. 2. Brain stem of decerebrate cat viewed from in front (diagrammatic) showing standard position of electrodes. *AA*, Anterior colliculi. *PP*, Posterior colliculi. *C*, Cut surface of brain stem. E_1 "Active" electrode; E_2, "Inactive" electrode.

We soon found that the size of electrical response was greatly influenced by the duration of the period of rest allowed between successive stimuli. Isolated single stimuli each following a rest of 5 or 10 seconds produced responses of practically uniform magnitude. If, however, a series of stimuli was applied with a frequency of four or five a second, the first response, assuming it followed a period of rest,

would be full-sized, but the remaining responses would be much smaller. The decrease in size between the first and second was marked, that between second and third less, and the decrease in subsequent responses little or none. In this respect the responses were strikingly similar to those of the motor nerve in the flexion reflex in the decerebrate animal, as recorded by Forbes and Gregg (10). A possible inference seems to

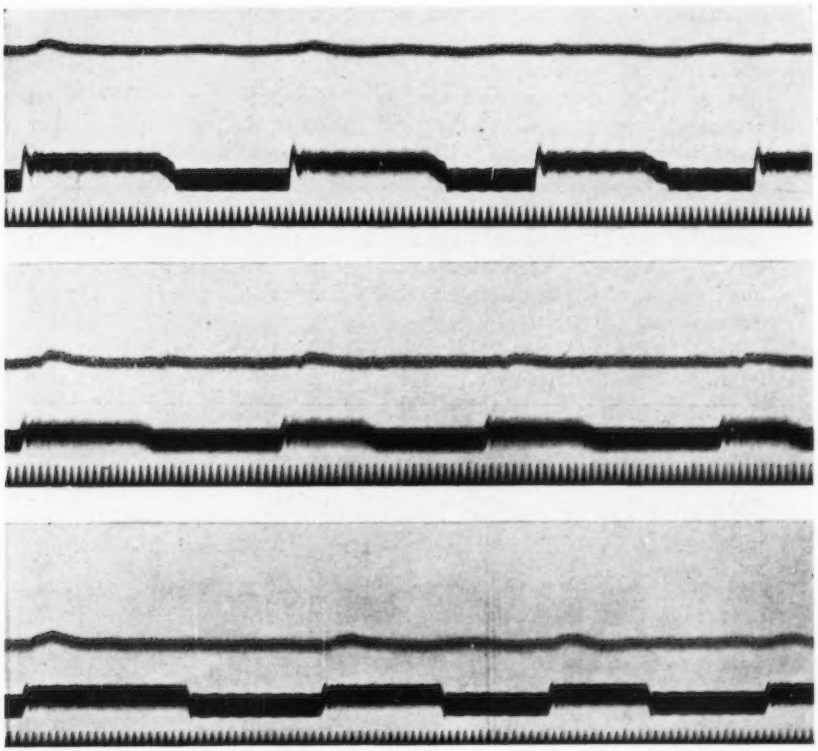

Fig. 3. Effect of repeated stimulation (see text). *A*, experiment 17. Leads on brain stem. String C; tension, 1 cm. excursion = 6.4×10^{-8} amp. Stimuli;—break shocks, 96 Z units; make shocks not determined. *B*, experiment 18. Leads on brain stem. String C; tension, 1 cm. excursion = 7.1×10^{-8} amp. Stimuli;—break shocks, 75 Z units. *C*, experiment 29. Leads on cerebellum. String C; tension, 1 cm. excursion = 6.7×10^{-8} amp. Stimuli;—break shocks, 36 Z units. Rise of signal magnet shadow shows break of primary current; fall shows make.

be that there is in each case a synaptic fatigue of rapid onset, but in each case it was not clear why a certain portion of the response remained apparently so highly resistant to fatigue. Typical examples of the response in the brain stem under this repeated stimulation are shown in figure 3, together with the result of the same procedure with the leads applied to the cerebellum. In order to avoid error arising from this reduction in the size of response on repeated stimulation we adopted as standard procedure the practice of allowing 5 or 10 seconds of rest before applying each recorded stimulus which was used to test the effect of ether anesthesia.

After the adoption of this method experiments were performed on seventeen animals in which the modification of the responses by ether was observed and recorded. In most of these, responses were recorded before the administration of the anesthetic, at various stages during the recovery and after recovery was complete. In several experiments this procedure was carried out two or three times; that is, the course of two or three successive etherizations was studied. Figure 4 illustrates the modification of the response by ether occurring in a few fairly typical experiments.

From figures 1 C, 3 and 4, it will be seen that in the typical response following the stimulus by an interval of about 0.01 second there is a

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Fig. 4. Galvanometer records showing effect of ether on brain-stem responses. Each horizontal row shows consecutively the progress of a single experiment. *E* signifies the point in the experiment at which ether was applied; *O*, the point at which it was withdrawn. The first observation of each row shows a typical reponse just before ether was given. The times of the other observations are listed with the application of ether as a starting point. Magnification of string, 580 diameter in all.

A, experiment 11. String C; tension, 1 cm. excursion = 5.6×10^{-8} amp. Stimuli;—nos. 1–5, 144 Z units; no. 6, 198 Z units. Times of observations;—2, 1 minute; 3, 3 minutes; "O", 4 minutes; 4, 5 minutes; 5, 12 minutes; 6, 26 minutes.

B, experiment 16. String C; tension, 1 cm. = 6.7×10^{-8} amp. Stimuli;—125 Z units. Times of observations;—2, 2 minutes; 3, 7 minutes; 4, 9 minutes; "O", 9 minutes; 5, 10 minutes; 6, 56 minutes.

C, experiment 17. String C; tension, 1 cm. = 6.4×10^{-8} amp. Stimuli;—122 Z units. Times of observations;—2, 1 minute; 3, $3\frac{1}{4}$ minutes; 4, 6 minutes; "O", $7\frac{1}{2}$ minutes; 5, 9 minutes; 85 minutes.

D, experiment 18. String C; tension, 1 cm. = 7.1×10^{-8} amp. Stimuli;—75 Z units. Times of observations;—2, $1\frac{1}{2}$ minute; 3, $3\frac{3}{4}$ minutes; 4, 6 minutes; "O", 6 minutes; 5, 16 minutes; 6, 24 minutes.

E, experiment 13. String C; tension, 1 cm. = 5.6×10^{-8} amp. Stimuli;—212 Z units. Times of observations;—2, 2 minutes; 3, 3 minutes; "O", $4\frac{1}{4}$ minutes; 4, $5\frac{1}{4}$ minutes; 5, 12 minutes.

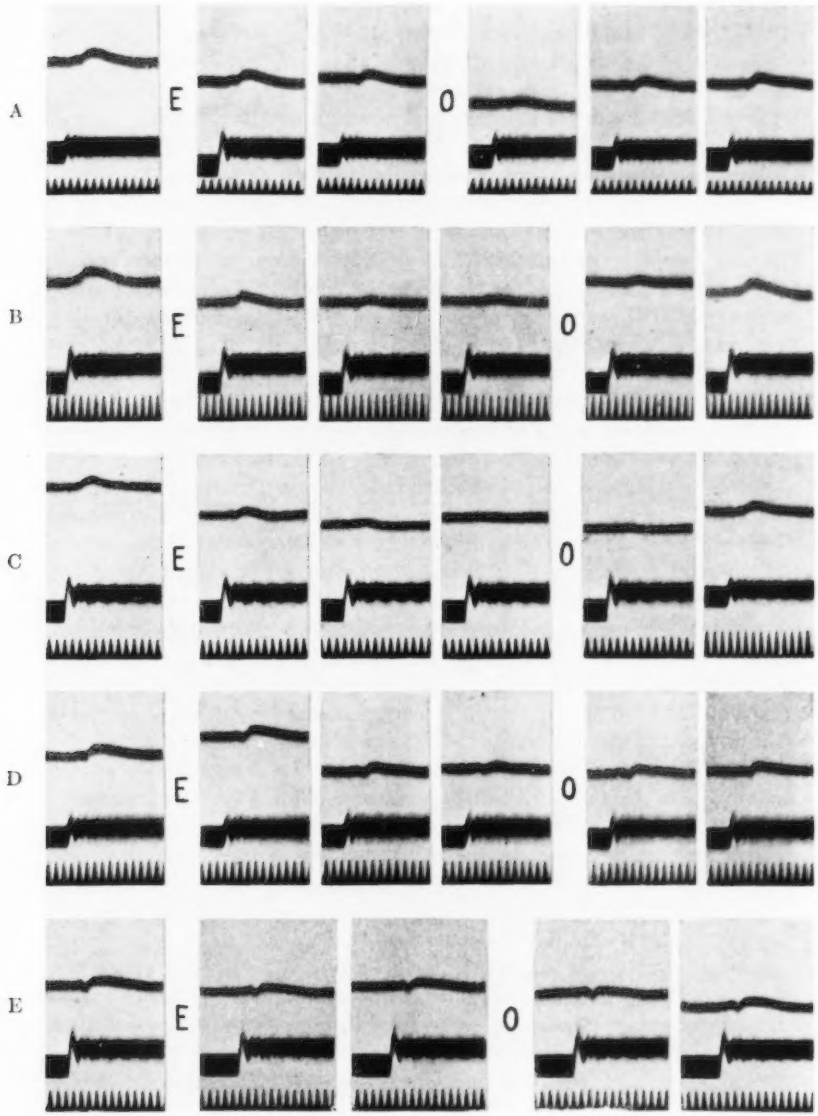

FIG. 4

small initial excursion, followed after a somewhat longer interval by a considerably larger one. The principal change attending the administration of ether is a gradual reduction in the size of the second excursion, the small initial one being comparatively little altered. In a later section we shall discuss the probable significance of the different parts of the curve. For the present we shall take as a measure of the effect of ether, the reduction of the major excursion.

A general survey of the results obtained with the standard procedure, described above, is shown in figure 5, in which the magnitude of the second (major) excursion is plotted against time in several typical experiments. Inspection of these curves and of the electrical records on which they are based strongly suggests the tentative conclusion that profound ether anesthesia greatly reduces the activity in those afferent paths lying in the vicinity of the electrodes.

Whenever in the experiments represented in figure 5 the disappearance and reappearance of the various reflexes were noted, the facts are indicated in the figure. Ether was generally administered till the respiration showed signs of impairment, usually 4 or 5 minutes. In general we found that the flexion reflex in response to a single shock disappeared between 1 and 3 minutes after the administration of ether was begun. Often at about the same time, but more often a few seconds later, the corneal reflex disappeared. These statements are based not only on the typical experiments represented in figure 5, but also on all the others. It has been mentioned in a previous paper (6) that the pinna reflex persisted under ether almost as long as respiration. This observation has usually been confirmed when the point was tested in the course of these experiments. The time of disappearance of the flexion and corneal reflexes was always much earlier than the disappearance of the pinna reflex, this usually lasting unimpaired for 4 or 5 minutes; i.e., till the respiration was beginning to weaken. Comparing the course of the reflexes with that of the galvanometric records, we found that the flexion and corneal reflexes usually disappeared at about the same time as the first reduction in the excursions, but sometimes they persisted till the reduction was considerable.

On withdrawal of ether the effect continued to become more profound, reaching its maximum in about 1 or 2 minutes. When recovery began, the reflexes generally reappeared in the inverse order, and their appearance was usually correlated with about the same magnitude of galvanometric excursion as their disappearance. In some experiments the excursions were distinctly smaller when the flexion reflex returned

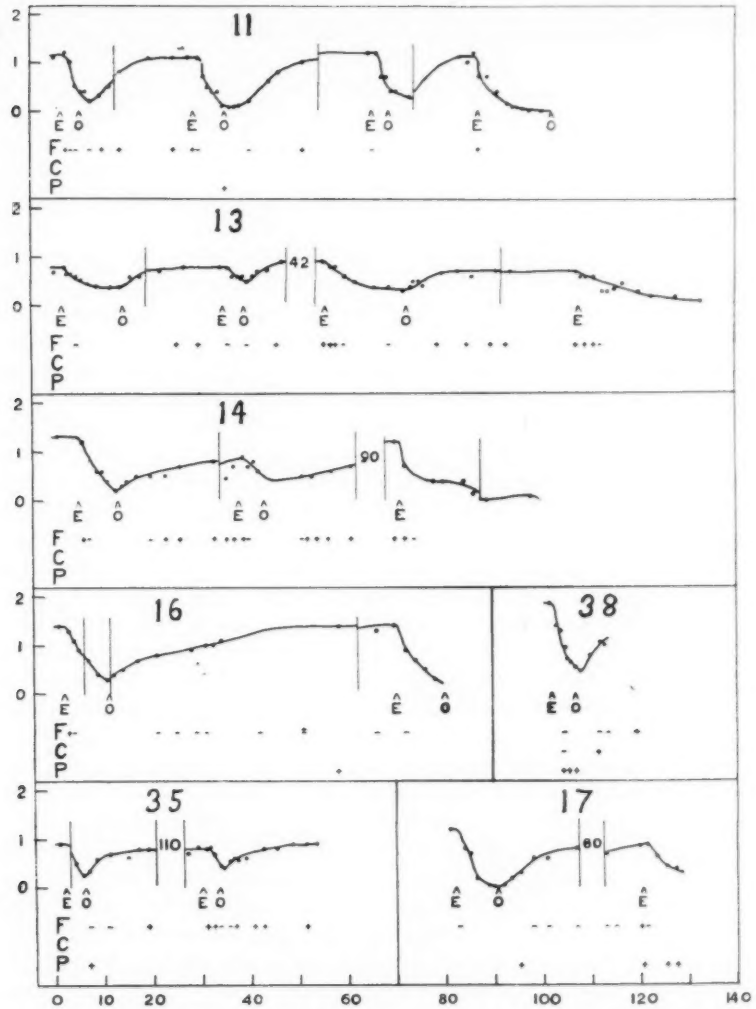

Fig. 5. Graphic record of results. Abscissae, time in minutes. Ordinates, major excursion in millimeters. Interruption of the curve by a vertical line means readjustment of electrode contacts, string tension or (in later experiments) amplifying system, thus altering the excursion independently of physiological change. Interruption by two lines with a figure between means a lapse of time of the number of minutes indicated. Application and withdrawal of ether indicated as in figure 4. Reflexes present (+) or absent (−), when noted, as follows;— *F*, flexion reflex; *C*, corneal reflex; *P*, pinna reflex (see text). The figure above each curve is the serial number of the experiment.

than when it disappeared, but this difference can hardly be set down as a constant finding. It will be seen from the figures that the return of activity in the nervous system was usually about two or three times as slow as its decline. The speed with which these functions returned was appreciably more constant than the speed of their disappearance. Since no strictly quantitative method of administering ether was employed, these comparisons merely mean that there was some variation in the rate at which the concentration of the drug increased in the blood, and that this was considerably more rapid than its subsequent rate of decrease. With respiration progressing normally we should expect the elimination of ether to occur at nearly the same rate in experiments performed under uniform conditions. The recovery from the anesthetic in these experiments in general fulfilled this expectation.

Analysis of the electrical response. Before the results described above can be properly evaluated, we must arrive at some idea of the significance of the different parts of the electrical response as revealed by the galvanometer. In particular, the fact that the second excursion of the string was always far more affected by ether than the first, makes it important to know what each excursion means, i.e., what underlying nervous activity is the cause of each.

In attacking this problem, it is of the greatest importance to bear in mind that direct contact of electrodes with the active tissue is not essential to the recording of action currents. Conversely, galvanometric excursions do not necessarily signify physiological activity in the tissues with which the electrodes actually make contact. This fact is well illustrated by the familiar practice of recording electrocardiograms by applying electrodes to the hands. All the tissues of the body conduct electricity to some extent and may therefore be regarded as extensions of the electrodes. What is necessary in order that the electric response of a given tissue may be registered in the recording apparatus is simply that the difference of potential arising in the tissue as a result of its activity shall set up lines of electrical force (and consequently of current flow) whose topographical arrangement is such as to cause a difference of potential between the two points on the body to which the electrodes are applied. When a nerve impulse transverses a nerve fiber its progress is marked by a transient state of lowered electrical potential of the active part with respect to the inactive portions both before and behind the disturbance. At a given moment the region on the body surface which is electrically nearest (i.e., connected by the path of lowest resistance) to the active point in the nerve will

have a lower potential than more remote portions of the surface, assuming all other tissues to be electrically inactive at the time. The intensity of this effect will, of course, be greatly reduced by the short-circuiting effect of other tissues surrounding the nerve and lying between it and the surface. A mass of tissue with very high resistance lying between the point of activity and the surface directly over it might cause the point of lowest potential on the surface to be more remote, and thus to disguise the position of the seat of activity. Nervous tissue has higher resistance than most other tissues; therefore in applying electrodes to such a complicated mass of nervous tissue as the brain stem with its complex relations to surrounding bony and vascular tissue, one must be guarded in drawing inferences as to the seat of activity from the recorded electrical response.

In seeking to place the seat of activity represented by each of the two excursions appearing in the majority of our records, we found little help from the topography of the brain stem and the position of the afferent paths. But from physiological considerations we were led to a working hypothesis.

It is known that some of the afferent fibers in the sciatic nerve extend centrally as far as the medulla oblongata. We had also learned from the preliminary experiments (6) that even the deepest general anesthesia fails to abolish the impulses in the peripheral nerve trunk. Presumably this is also true of the afferent axons within the spinal cord. It is more likely that the difference between nerve trunk and reflex arc, as regards the effect of ether (cf. 12, p. 80) is traceable to the synaptic region rather than to the mere passing of the fibers into the substance of the spinal cord. Therefore we should expect some of the impulses set up by the peripheral stimulus to penetrate as far as the medulla, even under the deepest general anesthesia.

In the cat the medulla is not very far from the points on the brain stem to which the electrodes were applied in our experiments. Therefore it is conceivable that impulses in the neurones terminating in the medulla might establish a large enough difference of potential in the electrodes to account for the initial excursion in the galvanometer. The persistence of this initial deflection in deep surgical anesthesia would be intelligible on this basis, and the disappearance of the second excursion, presumably representing activity in the second neurones in the chain, would likewise be intelligible if the drug acted, as Sherrington has inferred (12, p. 17), at the synapse.

The best way to test this hypothesis seemed to be to determine the time of arrival of the afferent impulses in the upper spinal cord or in the medulla by direct observation, and to compare it with the time of the first excursion in the brain-stem records from the same animal. The time of stimulation being shown in each case by the signal magnet, the comparison should be a simple matter, if the desired records could be obtained.

We supposed at first that it would be easier to expose the spinal cord in the upper thoracic region, without disturbing the vital functions, than to expose the medulla. We therefore performed several experiments in which after making records from the brain stem as usual, we exposed the spinal cord in the upper thoracic region, and applied the leading-off electrodes directly to it.

In six experiments this method was tried; in another in which decerebration had not been performed, the electrodes were likewise applied to the spinal cord, ether being withdrawn just enough to permit the appearance of the flexion reflex in response to test stimuli. The region exposed was usually between the scapulae, and in all cases between 5 cm. and 27 cm. from the occiput. In our first attempts we exposed the cord for several centimeters and, after taking the usual records from the brain stem, transected the cord at the anterior end of the exposed region, then lifted it out from the surrounding tissues and laid it on a thin hard rubber plate in order to insulate it and thus prevent the short-circuiting effect of the surrounding tissues. Electrodes were then applied to the lateral surface and cut end of the cord, and the usual stimuli applied as before to the sciatic nerve. The effect of this procedure proved extraordinarily destructive. Whereas the animals usually survived for hours after decerebration and exposure of the brain stem and even of the cerebellum, they usually died in less than 10 minutes after transection of the cord in this upper thoracic region; in no case did an animal survive this operation more than 20 minutes. In four of these experiments the electrodes were applied to the cord after it was cut, and photographic records were made. In three of these the records were made before death, and in one case just after. In only one case did the stimuli evoke reflex responses after electrodes were applied to the cut spinal cord. In none of them was there a measurable electrical response in the cord. In the one experiment in which there were reflex responses there appeared to be a very small deflection in the galvanometer following stimulation, but this could only be seen by looking at the film obliquely and was too small to serve for the desired measurement of latency.

In five of the seven animals whose spinal cords were exposed a different method of obtaining the records was tried. The cord was merely exposed and the electrodes applied to it *in situ*. In this way the destructive effect of transection was avoided. In two of these experiments the cord was not cut at all. In the other three both methods were employed, the electrodes being first applied to the cord *in situ*, and then the cord being dissected out and cut, and the electrodes reapplied, with the results already described in the previous paragraph. One difficulty presented by this procedure was that owing to the proximity of the heart to the electrodes the record was confused by an electrocardiogram. To overcome this difficulty we took repeated records, endeavoring to apply as many as possible of the stimuli at the intervals between the heart beats, so that any action current in the cord might be registered during a portion of the cardiac cycle when the string was tracing a steady base line. In two of these experiments no excursions correlated with the stimuli could be detected. In a third there was the possibility of a small excursion, but it was so small as to be doubtful. In the remaining two experiments, however, we obtained a considerable number of well-defined excursions which were evidently due to action currents in the cord. The direction of the excursions was in each case such as to indicate lowered potential at the electrode nearest the brain. This probably signifies that the disturbance traversed the distance between the electrodes so rapidly that only the second phase of the diphasic action current produced a measurable excursion; the string had not time to be appreciably displaced before the disturbance, reaching the second electrode, reversed the direction of the current. Therefore the excursion marks the arrival of impulses at the electrode nearest the brain.

The measurements of latency could be made accurately to within about 1σ. From one experiment, in which the electrodes were applied to the cord about 16 cm. behind the occiput, the latencies of five action currents were measured. They all appeared to be between 5 and 6σ, the average of the measurements being 5.3σ. Measurements of the first excursion previously obtained from the brain stem showed an average latency of 10.5σ. In the other successful experiment, the spinal cord being exposed for a small distance at each of two points, one 13 cm., one 37 cm. from the occiput, and one electrode applied to each opening, the latencies of six action currents were measured and found to average 5.0σ. In this experiment the first excursion previously obtained from the brain stem showed an average latency of 9σ.

From these observations we may conclude that the first excursion in the brain stem record occurs at a time when the in-coming impulses have, at least, passed into the cervical cord, and probably at a time when they have reached the medulla oblongata. But the exact portion of the afferent path whose activity was represented by this excursion was not yet identified.

The next step in the inquiry was to place one electrode directly on the medulla, and see if records could be obtained for comparison with those derived from the brain stem. We found it possible to remove part of the occiput and atlas, and expose the medulla without apparently disturbing the vital functions to any great extent. The procedure proved far less destructive than severing the spinal cord in the thoracic region.

One difficulty in the way of measuring accurately the latency of the excursions lay in the fact that they are small, at best, and of somewhat gradual onset, so that if the film was made to travel at a high enough speed to facilitate careful time measurements the galvanometric excursions became so flattened out in the record that the moment of onset could not be detected with precision. At the time when we decided to try taking records directly from the medulla, a method of amplifying galvanometric excursions with an electron-tube had become available in the laboratory (11). This enabled us to improve the technic of recording and determining the time intervals considerably. We were able to work with a fairly tight string and thus minimize the lag which results from using it very slack; and yet we were able to get large enough deflections to measure the moment of their onset on a high speed film with a fair degree of accuracy. Five successful experiments were performed in which the records were first taken from the brain stem as usual and then the electrodes were applied, one to the posterior surface of the medulla 3 to 8 mm. behind the cerebellum, the other to the posterior colliculus which had served for the "inactive" lead in the previous observations.

The method of amplifying with an electron-tube has already been described (11). It was modified in one respect for these experiments by the introduction of a precaution for the protection of the galvanometer. In order to obtain the greatest efficiency of amplification we regularly employ a negative "grid bias" of 1.5 or 3.0 volts (one or two dry cells in the grid circuit). If the grid circuit were suddenly opened under these conditions, the change in the tube resistance would probably cause so great a current in the galvanometer circuit as to break

the string. Since a slight motion of the decerebrate preparation might easily break the contact of one or both electrodes with the tissue, some means must be found to prevent this risk. For this purpose we introduced a shunt with a resistance of about 1 megohm, connecting the lead-wires from the non-polarizable electrodes. In this way it was impossible for the grid circuit to be broken by the dislodging of an

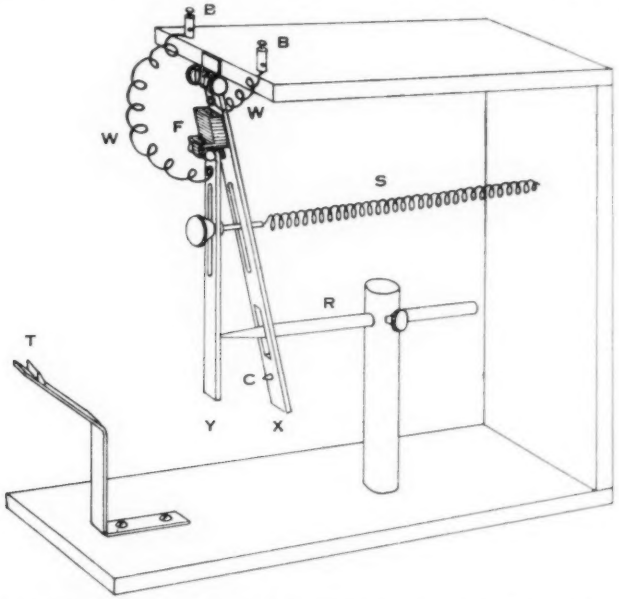

Fig. 6. Circuit-breaking key used in experiment 33 (fig. 7A). *BB*, binding posts. *WW*, wire connections. *F*, fiber hinge to insulate the two parts *X* and *Y*, when open. *S*, spring which operates key. *T*, trigger with catch which engages *X*, and when depressed, releases it. *R*, adjustable rod, which, protruding through opening in *X*, obstructs motion of *Y*, thus opening key. *C*, amalgamated copper contact at which circuit is broken.

electrode. The shunting effect of so large a resistance causes a slight reduction in the galvanometric excursions, but this is unimportant compared with the risk of losing the string.

In the first of these experiments made for the purpose of comparing the time relations of the responses from the medulla and brain stem we recorded the moment of stimulation on the film by breaking the primary circuit of the stimulating coil with a special key (fig. 6) which threw a

shadow on the film. The opening of the key is shown by the separation of two parallel lines which are the shadows of the two moving parts of the key (see fig. 7, A); this shows the time of stimulation more accurately than the signal magnet used in the earlier experiments. Close examination of the galvanometric record in this figure also reveals that at the time of stimulation the string regularly makes a brief, small excursion. This is due to the electrical artefact which is almost always rendered visible by electron-tube amplification. In subsequent experi-

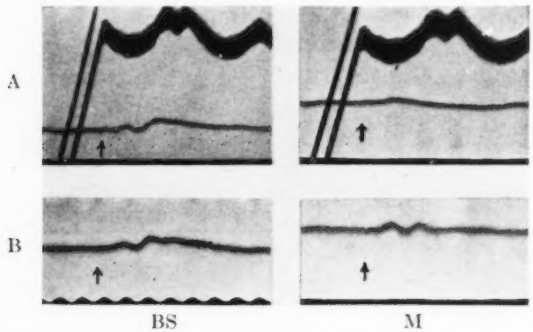

Fig. 7. Records showing by latency the identity of the first excursions in brain-stem and medulla responses. *BS*, brain stem; *M*, medulla.

A, experiment 33. Cambridge galvanometer. "String H," of gilded quartz, 2.5μ diameter, 12000 ohms resistance; magnification 300 diameters; tension, 1 cm. = 3.3×10^{-7} amp.; electron tube amplification. Speed of film in both observations, 30 cm. per second. The right hand oblique line is the shadow of the moving part, *Y*, of key (fig. 6), the bend in it showing time of stimulation.

B, experiment 39. Hindle galvanometer. "String 2," gilded quartz 1.5μ diameter, 25000 ohms resistance; magnification, 490 diameters; tension, 1 cm. = 2.0×10^{-7} amp.; electron tube amplification. Speed of film in both observations, 33 cm. per second.

The electrical artefact in each of the four observations is indicated by an arrow.

ments we relied upon the artefact to show the time of stimulation, and thus dispensed with the special key whose use proved rather clumsy. Figure 7 shows two fairly typical experiments in which the records obtained from the brain stem are compared with those obtained shortly afterwards from the medulla by the method described above. In each case the brain-stem record reveals much the same form as has appeared in previous experiments. In the records obtained from the medulla the first excursion may be recognized clearly as coincident in time with

the first excursion in the brain-stem records, but in the case of the medulla it is considerably larger than in the case of the brain stem.

In most of the medulla records (e.g., fig. 7, B and fig. 8) there is a well-defined second excursion corresponding in time with the second brain-stem excursion, but intead of being larger than the first, as in the brain-stem records, it is usually distinctly smaller than the first, and much smaller than corresponding excursions in the brain-stem records. In the experiment shown in figure 7, A this second excursion is absent in the medulla record.

Careful measurement of the latencies appearing in twenty-four separate observations from four experiments in which the artefact appeared clearly, confirmed the agreement of time intervals elapsing between the stimulus and the first and second excursions respectively

TABLE 1

Average latencies

EXPERIMENT	BRAIN STEM			MEDULLA		
	First excursion		Second excursion	First excursion		Second excursion
	Beginning	Peak	Peak	Beginning	Peak	Peak
33	8.5σ	13.5σ		9.0σ	13.5σ	
35	8.2σ	13.2σ	26.0σ	8.1σ	13.3σ	26.0σ
38	8.8σ	12.0σ	24.6σ	7.5σ	13.0σ	28.0σ
39	8.0σ	13.9σ	25.4σ	8.8σ	12.4σ	26.2σ

in the comparison of brain-stem and medulla records, within the limits of observational error. Table 1 shows the averages of these measurements for each of the experiments. The gradual onset of the first excursion rendered its beginning more difficult to place accurately than its peak. The agreement in the latencies of the peak of the first excursion in the two methods of recording is therefore the important result appearing in the table.

From these observations it may be reasonably inferred that the underlying activities giving rise to the corresponding excursions are the same in the two methods of recording, and that shifting the leads has resulted merely in changing the relative intensity of the electrical effects obtained in the galvanometer circuit.

In order to confirm the interpretation and to make certain of its application to the previous observations on the effects of ether, we etherized the animal as before while recording action currents from the

brain stem, then allowed the ether to be eliminated, and repeated the procedure while recording action currents from the medulla. This was done in four of the five experiments. Examples of the results in three of these are shown in figure 8. The observations shown in A and B confirm the results of previous experiments in that the principal change resulting from ether is a diminution of the second excursion; there is, however, an appreciable reduction in the first excursion as well, an effect barely discernible in some of the earlier records, but brought out more clearly by means of the electron-tube amplification. In figure 8 C, there seems to be a disturbance of the time relations under ether, the significance of which is not clear.

The significant fact which appears in these records is that the neural activity which gives rise to the first excursion and which is little affected by deep etherization, is that which causes the major electric response when the galvanometer is connected with the medulla. The direction of the excursion is such as to indicate lowered potential in the electrode on the medulla, and therefore, presumably, the seat of activity in that vicinity. It is therefore a reasonable inference that the peak of the first excursion marks the time when the majority of the nerve impulses pass under the electrode on the medulla. The fact that the second excursion, indicating negativity of the "active" electrode, is the major excursion when that electrode is applied to the brain stem, and is in the direction indicating negativity (lowered potential) in that electrode, leads to the inference that this excursion is caused by the arrival of the nerve impulses in the fillet in the vicinity of the cut surface of the brain stem.

The magnitude of the initial excursion recorded from the medulla presents an interesting contrast with the excursions (or usual lack of excursions) obtained when the leads were applied to the spinal cord. A possible explanation of the apparent increase in electrical activity when the disturbance reaches the medulla might be found in the large number of end branches in which the axons terminate. The effect of the multiplication of nerve impulses as each axon divides into many small branches and the disturbance thus becomes diffuse, might be to increase the action current which can be derived in the galvanometer.

In our working hypothesis we have assumed that, as Sherrington has argued, ether acts by blocking impulses at the synapse. The identification of the first excursion in the brain-stem records with the maximum disturbance in the medulla supports our hypothesis since, as has already been pointed out above, some of the peripheral neurones extend as far

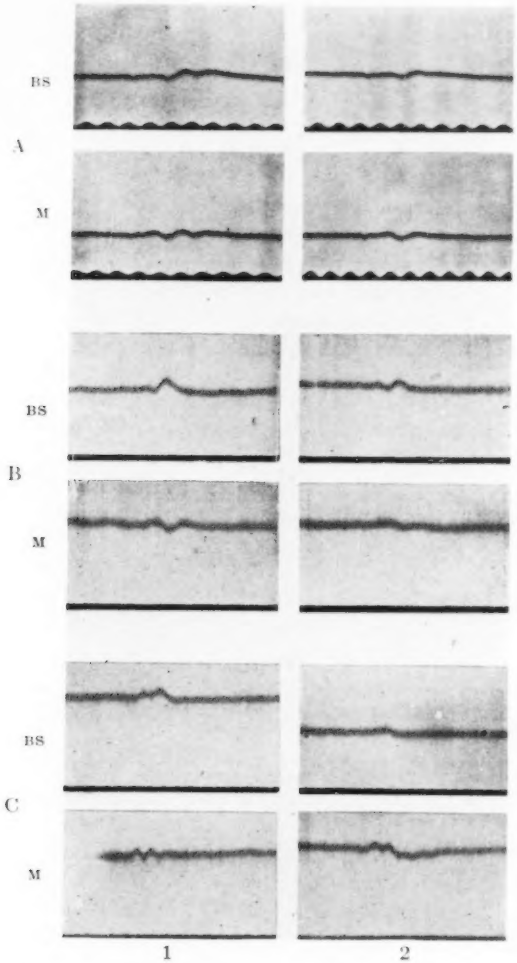

Fig. 8. Effect of ether on brain-stem and medulla responses compared. Electron tube amplification throughout. *BS*, brain stem; *M*, medulla.

A, experiment 35. Cambridge galvanometer. String H; magnification, 300 diameters; tension, 1 cm. = 5.5×10^{-7} amp. Tuning fork shadow shows time: each complete vibration = 0.01 second.

B, experiment 38. Hindle galvanometer. String 2; magnification 490 diameters; tension, 1 cm. = 2.7×10^{-7} amp. Speed of film in brain-stem records, 24 cm. per second; in medulla records, 30 cm per second.

C, experiment 39. Hindle galvanometer. String 2; magnification 490 diameters; tension, 1 cm. = 2.0×10^{-7} amp. Speed of film in all 17.5 cm. per second.

In each experiment observation *1* is without ether; observation under ether, *2*.

Note change of speed of film in *B*.

as that. The interpretation of the first excursion as depending on the multiplication of impulses as the axons branch, would explain the appearance of a well-defined maximum in the recorded electrical activity, and would harmonize with the view that interruption by ether occurs at the synapse, since branching occurs before the synapse is reached. The observed gradual onset of the first excursion is to be expected, for there must be some effect on electrode potential before the impulses reach the nearest point in their course to the electrode. The detection of this onset earlier in some of the medulla records than in the corresponding brain-stem records is also to be expected because of the closer proximity of the electrode to the seat of activity.

The slight reduction of the first excursion under ether in some of the experiments is interesting in connection with the discussion by Stiles (13) of the probable effect of attenuation of small end branches of axons, in offering to the surrounding fluids a large surface in proportion to their volume. According to a view tentatively proposed in a recent paper (14), that the difference between axon and synapse is only a matter of the degree of attenuation of the conducting fiber and its sheath, the slight reduction of the disturbance in the attenuated end branches and the blocking of impulses at the still more attenuated synapses, would be intelligible as depending on the degree of decrement established in these structures by a given concentration of ether in the blood.

According to the "all-or-nothing" law, if an impulse passed the synapse it would attain its full magnitude in the axon of the second neurone in the chain, provided this was not so affected by ether as to conduct with a decrement (15). Assuming this second axon to be no more affected by ether than those in a peripheral nerve, we should not expect a decrement here. Therefore it is probable that in the brain-stem records the reduction by ether of the second (major) excursion, which seems to be identified with the activity of the second neurone, means abolition of impulses in most of these neurones, depending on total blocking at the synapse; and the persistence of a small second excursion, even under deep anesthesia, means that in a few synapses the block is not complete. This is more probable than that the reduction measures an actual decrement in impulses which have passed the synapses and are traversing the second neurones.

Obviously these observations do not warrant any conclusion as to the physical nature of the synapse, the extent to which they harmonize with the proposed view is merely mentioned incidentally. Our results

do, however, strongly support the view that whatever the nature of the synapse, it is there that the principal blocking effect of ether occurs. Since the total volume of impulses traversing the afferent neurones in the fillet is so greatly reduced under surgical anesthesia, it is likely that further reduction occurs at the synapses in the thalamus, and consequently very few impulses reach the sensory cortex as compared with those reaching it under similar stimulation in absence of ether.

Application of electrodes to cerebellum. In six experiments the electrodes were applied to the cerebellum. This was done chiefly because observations on cells in the cerebellum have been made the basis of some of Crile's conclusions, with the validity of which we are concerned. In the first of these experiments the dura mater was at first not cut, the electrodes being applied to its outer surface. Several positions on the cerebellar surface were tried. The excursions in the galvanometer were extremely small, but they were sufficiently large and consistent to make possible the following statement. When the electrodes were symmetrically placed on the right and left lateral lobes, the excursion indicated lowered potential in the electrode on the opposite side from the stimulated nerve. When the electrodes were applied to the posterior and anterior ends of the vermis, the lowered potential appeared at the posterior end. When the electrodes were applied obliquely, that is, one to the right anterior lobe and the other to the left posterior lobe, for example, the excursions seemed to show that the lowered potential occurred at the posterior electrode, whether it was on the same side as the stimulated nerve or on the opposite side. In this animal the excursions were much smaller than those obtained from the brain stem in the usual manner. When the dura mater was cut and the electrodes applied directly to the surface of the cerebellum, the excursions increased slightly, but were still very small. Subsequent experiments confirmed the conclusion that the posterior electrode in general becomes negative in response to stimulation; but the observation in which the electrodes were symmetrically placed on opposite sides was not repeated in other preparations and therefore must not be given much weight.

In the remaining five experiments with the cerebellum, the electrodes were applied as follows: One, the "active" electrode, was applied to the posterior portion of the cerebellum, the other electrode was applied to one or other of the colliculi. In the three experiments shown in figure 9 the active electrode was on the posterior crus secundum on the same side as the stimulated nerve, the other electrode being on one of the

colliculi on the opposite side. In the two experiments shown in figure 10 the active electrode was applied to the posterior part of the cerebellum as explained in detail in the legend, the other electrode being applied to the posterior colliculus on the same side as the stimulated nerve; that is, being left in the standard position used in making the brain-stem record. In preparation 27 (fig. 9, A) the excursion of the galva-

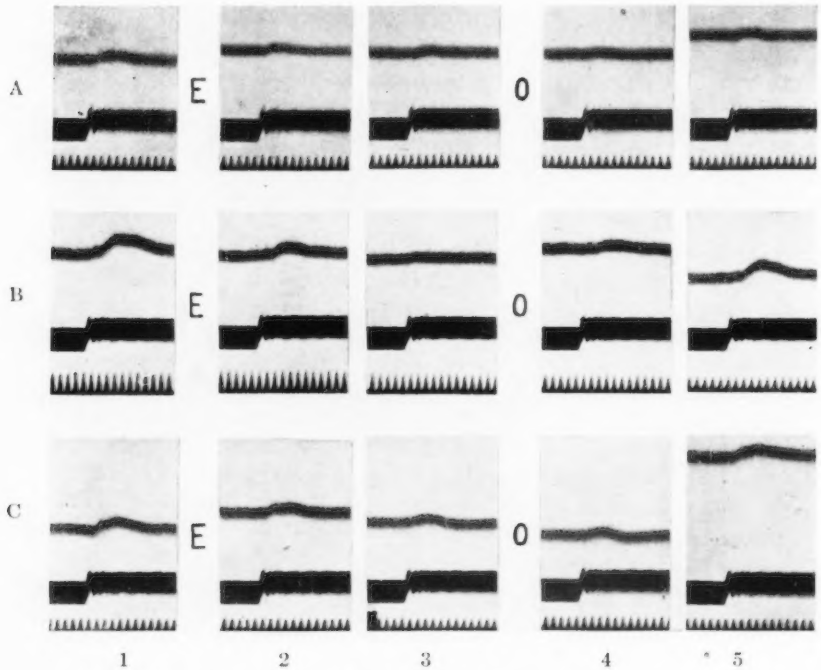

Fig. 9. Effect of ether with leads on cerebellum. Notation as in figure 4. String C in all; magnification 580 diameters. Cambridge galvanometer.

A, experiment 27. Tension, 1 cm. = 6.4×10^{-8} amp. Stimuli 43 Z units. Times of observation after ether was applied;—2, 1 minute; 3, 4 minutes; "O", 6½ minutes; 4, 8½ minutes; 5, 23 minutes.

B, experiment 28. Tension, 1 cm. = 5.9×10^{-8} amp. Stimuli, 48 Z units. Times of observations;—2, 1 minute; 3, minutes: "O", 7½ minutes; 4, 16½ minutes; 5, 49 minutes.

C, experiment 29. Tension, 1 cm. = 6.7×10^{-8} amp. Stimuli, 50 Z units. Times of observations;—2, 1 minute; 3, 5 minutes; "O", 5 minutes; 4, 9 minutes; 5, 32 minutes.

nometer obtained from the cerebellum was distinctly bigger than that obtained from the brain stem. In the other two shown in this figure, brain-stem records were not taken.

The effect of ether was studied in the three preparations shown in figure 9 in the same way as was done with the usual procedure of recording from the brain stem. The results were essentially the same, as is shown in figure 9. If any difference is to be seen in the results of the two procedures, it is that the reduction in the size of excursions by ether seems to be slightly more marked in the case of the cerebellum. Figure 10 shows the results of two of the more recent experiments in which amplification with the electron tube was employed and the re-

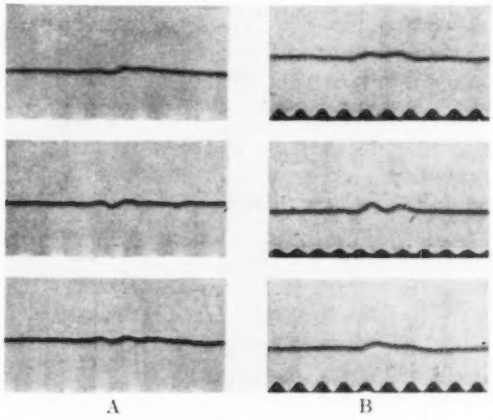

Fig. 10. Records showing correspondence in time relations of the two excursions in brain-stem, *BS*, cerebellum, *C*, and medulla, *M*, responses. Cambridge galvanometer, String H, magnification 300, electron tube amplification in all.

A, experiment 35. Tension, 1 cm. = 5.5×10^{-7} amp. Electrodes applied as follows; *BS*, standard arrangement (fig. 2); *C*, "active" lead on left posterior portion of cerebellum; *M*, "active" lead on medulla 4 mm. behind cerebellum; "inactive" lead on rt. post. colliculus in all.

B, experiment 36. Tension, 1 cm. = 4.2×10^{-7} amp. Electrodes as follows: *BS*, standard arrangement; *C*, "active" lead on posterior surface of cerebellum in median plane; *M*, "active" lead on medulla 3 mm. behind cerebellum; "inactive" lead on rt. post. colliculus in all.

In both experiments stimuli applied to right sciatic nerve; in *A*, 164 Z units; in *B*, 178 Z units.

In all observations, speed of film 32 to 33 cm. per second.

cords taken on a high-speed film to show the time relations accurately. It will be seen that the two excursions which we have already noted to have been constantly present in both brain-stem and medulla records are also present in the cerebellum records. As in the other two cases the difference seems to be one of the relative magnitude of the two excursions. The electrical records obtained by the three modes of leading off seem to be in the main expressions of the same series of neural activities in each case. It may be that these activities are only those of the chains of neurones leading directly to the brain stem and that the cerebellum acts merely as an inert conductor, or it may be that the cerebellum contributes to the disturbance recorded by all three methods. The increased magnitude in some of the earlier experiments in applying the leads to the cerebellum seems to indicate that this part of the brain, at least in some cases, contributes something to the total disturbance.

CONCLUSION

Returning to the consideration of the problem as outlined at the beginning of this paper, we are now in a position to give some sort of answer to the question whether ether anesthesia does protect the brain from the incoming stream of nerve impulses which is supposed to exert a damaging effect if allowed to enter the brain unchecked. Recourse to the action current, which is the most direct known index of nerve activity, reveals the following facts: Light ether such as first abolishes the corneal reflex, for example, causes a diminution in the electrical disturbance occurring in the brain stem and probably arising in the second neurones of the chain leading to the cerebral cortex. Since the individual nerve impulse probably attains its full magnitude in the axon after passing a synapse, and is thereafter conducted without decrement, it is probable that the observed diminution depends on the total blocking of impulses at some of the synapses between the first and second neurones of the chain, the block in the remaining synapses being incomplete. Further diminution as the anesthesia deepens probably means an increase in the percentage of synapses at which the block is complete. With deep surgical anesthesia the reduction in the total electrical disturbance is very great, sometimes amounting almost to abolition. From this observation we may conclude that very few nerve impulses pass the block established by ether in the first synapses of the afferent path leading to the brain, and therefore ether is of great value in protecting the brain from such damage as may be done by afferent impulses. In general the results harmonize well with

Sherrington's view that the synapse is the point at which the effect of the anesthetic occurs.

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COMPARISON OF PENTABROMACETONE METHOD, AND SALANT AND WISE'S METHOD FOR CITRIC ACID DETERMINATION IN URINE

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Applying the "pentabromacetone method"[1] for citric acid determination (1), Amberg and McClure (2) were the first to note the presence of citric acid in the urine of normal children and adults. They also obtained positive tests for citric acid in normal urine with the method of Salant and Wise[2] (3), which is based upon Denigès' reaction.

[1] Briefly the pentabromacetone method, as we applied it, is: To 50 cc. of filtered urine, 1 cc. of dilute H_2SO_4 is added and bromine vapor poured in. The clear solution, to which KBr and strong H_2SO_4 are added, is heated in a water bath at 50 to 55°C. for 5 minutes, after which 5 per cent $KMnO_4$ solution is added slowly. $MnO_2 \cdot H_2O$ and the excess bromine are removed by addition of $FeSO_4$ acidified with H_2SO_4. The precipitate obtained is weighed in a Gooch crucible, after drying 24 hours in a desiccator. It is then washed with acetone which dissolves out the pentabromacetone very quickly. After this, the crucible is again dried and weighed. From the pentabromacetone, we calculate the quantity of citric acid present, adding 5 mgm. in each case, because of an undeterminable fraction, which Amberg and McClure (2) had found to exist.

At times the acetone-insoluble part of the precipitate was considerable, as much as 9.8 mgm. Thus far the nature of this residue has not been determined.

[2] Salant and Wise's method, as applied by us is as follows: From a specimen of urine, which if not already acid to litmus is made so by addition of HCl, three portions of 10, 15 or 20 cc. each (quantity selected so that weight of Denigès' precipitate fell well within the limits of the table of Salant and Wise) were taken. These portions were evaporated nearly to dryness over water bath; volume of each was made up with water to 5 cc.; sufficient 10 per cent barium acetate added to insure excess of barium ion. Amount of barium acetate required was determined by a test on one of the three portions which was then discarded. Remaining two portions were made faintly alkaline with $Ba(OH)_2$; three volumes of 95 per cent alcohol were added; the mixtures allowed to stand an hour; centrifuged and the supernatant liquid poured off. The sediment was twice washed by adding 30 to 40 cc. of 50 per cent alcohol, stirring, centrifuging and discarding the supernatant fluid. The precipitate was treated with 10 drops of syrupy H_3PO_4

For the purpose of comparing the accuracy and practicability of these two methods for quantitative work in urine, we applied them to the determination of citric acid:

I. In normal urine
   a. Specimens from a series of children of various ages.
   b. Specimens from the same child on successive days.
II. In normal urine to which known quantities of citric acid, in the form of sodium citrate, had been added.

The results of the experiments for the comparison of the two methods applied to normal urine from a series of children are tabulated, as shown in table 1, *a*.

These children were chiefly orthopedic patients who were afebrile at the time when the urine was collected. The figures give some idea of the 24-hour excretion of citric acid during childhood. It is difficult to obtain 24-hour collections of urine from young children and, as Amberg and McClure (2) stated, the pentabromacetone method for quantitative determination of citric acid in urine is not yet as accurate as it might be.[3] For these reasons the figures given can only be regarded as approximate. The results obtained by the two methods show poor agreement in a considerable number of cases.

The data of the experiments for the comparison of the two methods when applied to the 24-hour specimens of normal urine, collected on successive days, are shown in table 1, *b*.

and transferred to a test tube by a jet of hot water. Residual alcohol was driven off by boiling and volume brought to approximately 10 cc. by evaporation. After addition of 0.7 cc. of Denigès' reagent, the mixture was heated to boiling; cooled; volume made up to 12 cc. and filtred. To one-half of filtrate, 0.35 cc. of Denigès' reagent was added and the volume brought to 6 cc. Solution was heated to boiling and 2 per cent $KMnO_4$ added drop by drop until a persistent pink or deep brown color appeared. Mixture was decolorized by three drops of H_2O_2, heated to boiling, cooled, and filtered on a weighed Gooch crucible. The precipitate was washed with water, then alcohol, and finally ether, as directed by Salant and Wise; dried at 90 to 100°C., and weighed. Using the equivalent of sodium citrate, as obtained from Salant and Wise's table, we computed the 24-hour excretion of citric acid.

The figures for citric acid, shown in the tables, for both the pentabromacetone method and Salant and Wise's method represent the average of duplicate determinations.

[3] An improvement of the pentabromacetone method, developed subsequently, will be published elsewhere by McClure.

TABLE 1. *a*

Quantities of citric acid found by the two methods in urine from children of various ages

NUMBER	AGE	URINE IN 24 HOURS	SPECIFIC GRAVITY	CITRIC ACID EXCRETED IN 24 HOURS	
				Pentabromacetone method	Salant and Wise's method
		cc.		*mgm.*	*mgm.*
1	7 months	320	1008	44	52
2	9 months	365	1009	96	80
3	14 months	594	1008	206	253
4	14 months	325	1007	53	52
5	15 months	380	1009	51	73
6	17 months	630	1007	134	130
7	25 months	222	1027	76	115
8	27 months	225	1032	213	180
9	33 months	190	1026	49	78
10	3¼ years	375	1019	99	91
11	3¾ years	176	1026	64	51
12	3 7/12 years	304	1021	149	146
13	4 1/12 years	265	1033	131	139
14	4¼ years	490	1014	102	150
15	4¾ years	375	1026	132	145
16	4¾ years	350	1030	254	152
17	5⅔ years	250	1030	127	121
18	5 1 1/2 years	227	1022	30	37
19	5 1 1/2 years	425	1019	134	102
20	6 1/12 years	850	1011	112	238
21	7 years	425	1015	182	167
22	7 1/2 years	550		79	98
23	7½ years	300	1025	112	84
24	7¾ years	450	1018	209	143
25	8¼ years	500	1020	296	294
26	8⅓ years	460	1021	230	247
27	8½ years	550	1016	154	165
28	8 1 0/2 years	425		82	92
29	9 years	650	1009	124	140
30	9½ years	890	1008	192	198
31	9½ years	795	1011	239	231
32	9½ years	740	1021	431	258
33	10 2/2 years	720	1017	269	259
34	10½ years	305	1026	88	88
35	10⅚ years	560	1015	114	139
36	11⅓ years	275	1033	257	190
37	12 years	522	1019	283	156
38	13 years	510	1024	240	187
39	14 years	1005	1017	241	177

TABLE 1, *b*

Quantity of citric acid from 24-hour collections of urine of a child aged 6 years and 2 months, collected during two periods of 3 days each

DATE	URINE IN 24 HOURS	SPECIFIC GRAVITY	PENTABROMACETONE METHOD	SALANT AND WISE'S METHOD
	cc.		*mgm.* citric acid	*mgm.* citric acid
4/18/18	615	1016	241 0	196 0
4/19/18	1100	1008	239 8	234 4
4/20/18	400	1016	135 2	124 0
4/24/18	480	1011	128 6	120 0
4/25/18	910	1006	200 2	212 0
4/26/18	750	1009	210 0	236 0

The excretion of citric acid was fairly uniform on 4 days. During 2 days the quantity of urine collected was low, as was also the amount of citric acid estimated.

For the purpose of controlling the accuracy of the two methods, we added known amounts of sodium citrate to normal urine and determined what amounts of citric acid could be recovered. The results are set forth in the following table:

TABLE 2

Quantity of citric acid from normal urine to which known amounts of sodium citrate had been added. Table gives milligrams of citric acid per 50 cc. of urine

NUMBER	PENTABROMACETONE METHOD				SALANT AND WISE'S METHOD			
	Native urine	Native urine plus 13 6 mgm. citric acid (as sodium salt)	Recovered		Native urine	Native urine plus 13 6 mgm. citric acid (as sodium salt)	Recovered	
			Amount	Per cent			Amount	Per cent
1	18.7	30.0	11.3	83	15.5	21 0	5 5	40
2	13.4	26 2	12 8	94	15.3	22 5	7 2	53
3	12.8	24.7	11.9	88	14.3	26 5	12 2	90
4	20.6	33 1	12.5	92	15 1	25 1	10 0	74

It is seen that in all experiments, except the third, a larger percentage of the added citrate is determined by the pentabromacetone method.

SUMMARY

Comparison of the results obtained by the two methods for the determination of citric acid in urine shows: 1st, that out of 39 cases, reported in table 1, *a*, there was fair agreement in 15 cases; 2nd, that there was better agreement in the case reported in table 1, *b*.

In the control experiments reported in table 2, where known quantities of sodium citrate were added to normal urine, the pentabromacetone method gave much better results than Salant and Wise's method in 3 cases. In the fourth case the results by the two methods showed close comparison.

It would seem that, for quantitative work in urine, the pentabromacetone method of citric acid determination is preferable.

We wish to acknowledge our indebtedness to Dr. Samuel Amberg for his advice and counsel throughout this work.

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THE DISTRIBUTION OF VITAMIN B IN THE WHEAT KERNEL[1]

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Previous investigations have shown that the wheat kernel contains a considerable quantity of vitamin B and that highly milled "patent" or "white" flours are very poor in this factor. The actual location of the vitamin has, however, been a subject of controversy. Commercial wheat embryo preparations have become one of the chief sources of vitamin B. (McCollum and Davis, 1915; McCollum and Kennedy, 1916; McCollum, Simmonds and Pitz, 1916; Daniels, Byfield and Loughlin, 1919.) In spite of the recognized content of such materials, the question has been raised whether the embryo contains all or even most of the vitamin, and if not, where the remainder is located. Doubt has also been expressed as to whether the vitamin is contained in the embryos proper or in the other substances present as an admixture in the milled product.

Chick and Hume (1917) stated: "In the wheat grain, the antineuritic vitamine is concentrated mainly in the germ or embryo; it is also present to a less degree in the bran (pericarp and aleurone-layer), probably in the aleurone-layer."

Voegtlin and Meyers (1918) gave their opinion that "the antineuritic vitamine seems to reside in the peripheral layers and the germ of these seeds," but changed this the following year to a statement that "the germ or embryo of the wheat contains *all* of the antineuritic vitamine" and (1920), "the aleurone cells are not the seat of the antineuritic vitamine. The substance resides in the portion containing the germ and probably within the germ."

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[1] The data in this paper are taken from a dissertation presented by Marion Bell to the faculty of the Graduate School in Yale University, 1922, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A preliminary account of these experiments was published by Marion Bell and Lafayette B. Mendel: The Distribution of Vitamin B in the Wheat Kernel. Proc. Soc. Exper. Biol. Med., xix, May, 1922.

Stammers (1921), on the other hand, found in his experiments "no symptoms which would give rise to the opinion that the antineuritic properties of the bran were inadequate."

Osborne and Mendel (1919) stated their findings as follows: "*Commercial wheat embryo* is rich in the water-soluble vitamine. Commercial bran and flour contain much less of this important food factor. The *pure embryo*, carefully separated from all other parts of the seed and used as the sole source of vitamine suffices to maintain young rats, but fails to promote their growth, even when supplied in quantities equal to the amount of pure embryo contained in such quantities of the commercial embryo as were sufficient to promote full normal growth. Wheat kernels from which the embryo has been carefully removed are still rich in water-soluble vitamine."

In view of the importance of the concentration of vitamin B in various wheat products and the diversity of opinions regarding the distribution of this factor in the grain, it seemed worth while to carry on further investigations in this matter. The present experiments were therefore initiated with the hope of obtaining some clear-cut evidence that would settle the question definitely. Mice were chosen as the experimental animals, since their food intake is comparatively small and they give results rapidly. These considerations are important especially when it is desired to test hand-dissected portions of the grain, the preparation of which involves a large amount of labor.

TECHNIC. The mice used in these experiments, a healthy stock, were kept in either the square cages with the special feeding device used by Mitchell (1922), or the cylindrical rat cages described by Ferry (1920). The mice were weighed twice and food once each week.

Paste foods were used in all cases. The standard diet of which all others were modifications was that used by Mitchell and Mendel (1921). It had the following composition:

	per cent
Casein	31
Corn-starch	38
Salt mixture	7
Lard	14
Butter fat	10

When wheat embryos or yeast were supplied as a source of vitamin B, the standard food was used as just described, the embryos or yeast being offered in addition each day. When the entire wheat kernel, bran, endosperm or milling products which needed to be used in large quantities were being tested, they were incorporated into the paste food. In such cases the quantity of protein in the particular per-

centage of wheat product being used was calculated and the casein correspondingly diminished. The remainder of the wheat was allowed to supplant an equivalent amount of starch. In a few cases where the wheat food tended to be too crumbly, the content of lard was increased 1 or 2 per cent at the expense of the starch.

Typical curves of growth of some of the experimental mice are represented on the accompanying charts.[2] Curves of the normal rate of growth for mice recorded by Thompson and Mendel (1918) were used for comparison in all cases and are represented on the graphic charts by a broken line. Control mice on standard food and yeast compared favorably with those of Thompson and Mendel.

The entire wheat kernel. Before taking up the study of the vitamin B in the different structural parts and milled products of wheat it was necessary to determine the approximate concentration in the entire berry, in order to have a standard for comparison. For this study two varieties of wheat were obtained: Marquis spring wheat from Peter Henderson, New York, and Minnesota winter wheat from the American Institute of Baking, Chicago. The method of procedure in both cases was as follows: The wheat was ground quite fine by means of a nixtamall mill and the product well mixed to avoid as far as possible any separation of different grades of material. A series of foods was then made up in which the ground wheat was substituted for different percentages of the casein and starch. The following table gives the formulae for such a series:

	PERCENTAGES						
Wheat	60	40	30.0	20.0	15	10	0
Casein	23	26	27.0	28.5	29	30	31
Corn-starch		4	12.5	20.5	25	29	38
Salt mixture	6	6	6.5	7.0	7	7	7
Lard	1	14	14.0	14.0	14	14	14
Butter fat	10	10	10.0	10.0	10	10	10

All these foods contained practically the same quantity of vitamin A, salts and proteins, and the same calorific value except for the first in which the fat was diminished to allow for the extra starch in the large quantity of wheat. The one variable factor most likely to influence growth was thus the vitamin B.

[2] Full records and charts of all animals used may be found in the dissertation of M. Bell, Yale University, 1922.

Two or more mice were fed on each food to be tested, with the purpose of determining just what percentage of wheat was the minimum necessary for growth at a normal rate. The results of these experiments will be discussed later.

Milling products of wheat. The milling products of wheat used in these experiments were provided by the American Institute of Baking, and have been described by Assistant Director C. B. Morison of the Institute as follows:

> The fractions of wheat are in the order of grade as follows: (1) patent flour, (2) first clear, (3) second clear, (4) low grade flour, (5) standard middlings, (6) bran.
>
> These notations were used to simplify the classification of the fractions. The flow sheets of the gradual reduction systems of milling from the large mills sometimes show as many as forty or more streams. For example, we may find them in practice marked as 1st, 2d, 3d, etc., break four; 1st, 2d, 3d, etc., middlings; chunk germ flour; 1st, 2d, 3d, etc., tailings, and various other notations. It is a complicated matter to follow out these various streams to their final commercial products.
>
> The sample designated "patent flour" is comprised of the various streams of "middlings" derived largely from the endosperm of the kernel. The loose use of the term "middlings" in practice is probably the cause of much confusion. Expressions such as "germ middlings," "flour middlings," "standard middlings," refer to distinct commercial products, sold as cattle foods and not necessarily to the products from the endosperm.
>
> The "first clear flour" usually contains endosperm, aleurone cells, aleurone membrane, episperm, endocarp, epicarp and epidermis. Millers usually aim to mill no more than ten per cent of the wheat into the first clear.
>
> The "second clear" is of lower grade than the "first clear" and contains more of the non-endosperm tissues.
>
> The "low grade flour" contains more of the non-endosperm tissues than the second clear. First and second clears are used to some extent by the baker, but more goes into cracker making. This statement also applies to the low grade flour.
>
> The "standard middlings" contains practically all of the germ. This term is recognized to describe a cattle feed. In other words it is composed of what is commonly known as "shorts" and "red dog."
>
> The bran sample needs no explanation.
>
> The patent flour should contain no appreciable germ. The first and second clear also should be comparatively free from this tissue. The low grade flour contains little, and the standard middlings *practically* all of it. The "bran" ought not to contain much germ.

One small lot of wheat was milled especially for the purpose of these experiments so that the different products would be comparable in coming from the same wheat. Kjeldahl total nitrogen determinations

were made on each sample in order to secure data for calculating the percentage of protein in the product and therefore the quantities of casein and starch to be replaced in the foods thereby. Different percentages of each product were tried with the object of determining the minimum quantity necessary to supply sufficient vitamin B for normal growth By comparison of the adequate minimum of a given product with that of whole wheat the relative concentration of vitamin B in that product could be estimated. The approximate percentage of the total vitamin B milled into the particular flour could then be calculated by multiplying the relative concentration of vitamin by the proportion of the whole grain represented by the flour.

Hand-dissected portions of the wheat kernel. In order to remove the embryo completely from the endosperm it was found to be very helpful to soak the grains in water. To get the desired effect at room temperature it was necessary to soak for some time, thus allowing a possible incipient sprouting and change in the vitamin content. Pouring the grains into boiling water seemed to soften them more quickly and obviated any danger of sprouting. Chick and Hume (1917), Daniels and McClurg (1919) and others have demonstrated that boiling for an hour has little if any deteriorating effect on the content of vitamin B in foods, if the reaction is not alkaline. It is therefore unlikely that the minute or two of boiling to which the wheat was subjected in the softening process had any deteriorating effects.

The grains swelled in the water and the embryos could be readily removed from the endosperm with the point of a scalpel. The yellow embryo could be easily distinguished from the white endosperm, and if the separation was carefully made by pokng rather than by cutting, each part presented a smooth shining surface very different from the rough starchy appearance of the endosperm if it is cut into.

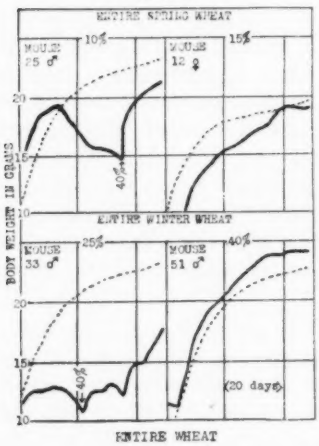
The attempt was made at first to remove every little particle of bran from the embryos, but when it was found that even 6 per cent of bran in the diet had no significance at all as a source of vitamin B, and since the total amount of wheat embryos used in any experiments, to say nothing of the bran associated with it, rarely approached that quantity, it did not seem possible that tiny portions of bran adhering to the embryos could introduce any appreciable source of error. Therefore, since the removal of these particles greatly increased the labor of this already difficult preparation, the traces of bran were allowed to remain. Every precaution was taken, however, to make a clean separation from the endosperm. The embryos were dried, and weighed daily portions were fed to mice in addition to the standard diet.

The *deëmbryonated grains* were dried either immediately or after being first cut into two portions—the "head-end" or end from which the embryo had been removed, and the "tail-end" or end farthest from the embryo. These products were dried and ground, and various percentages were mixed in foods as in the case of the milled products.

In one set of experiments the dry grains were cut in half crosswise with a sharp knife without previous soaking, and the two ends used without removal of the embryo.

CONCENTRATION OF VITAMIN B IN THE ENTIRE WHEAT KERNEL. *Marquis spring wheat.* Experiments were performed with 60, 40, 30, 20, 15 and 10 per cent respectively of entire spring wheat in the diet.

CHART I



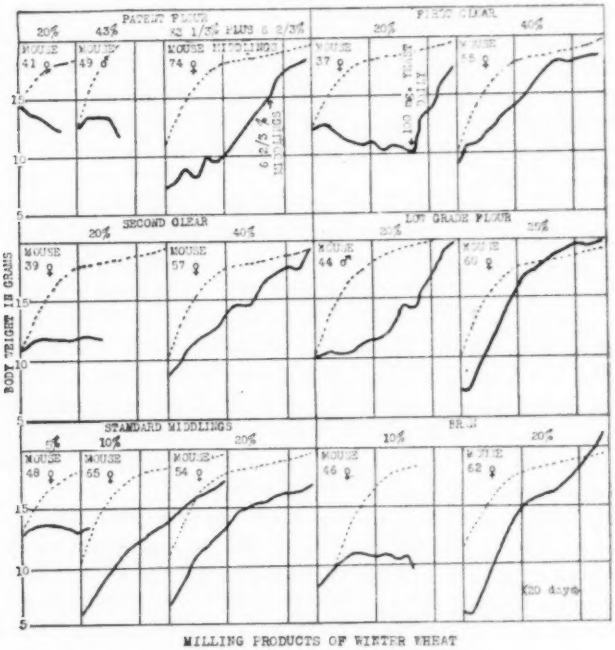
In all cases where the food contained 15 per cent or more the mice grew well and maintained a healthy appearance. When the diet contained only 10 per cent of wheat, the mice grew for a time, but then lost weight and showed characteristic symptoms of malnutrition. (E.g., mice 52 ♂, 12 ♀; chart I.) It seems, therefore, that 10 per cent of this wheat in the mixture selected is not sufficient to supply an adequate quantity of vitamine B, but that 15 per cent or more fulfils the requirements of growing mice in diets of the composition and caloric value indicated.

Minnesota winter wheat. The sample of winter wheat used was found to be poorer in vitamin B than the spring wheat. Twenty-five per cent of whole wheat failed to promote growth, but an increase to 40 per cent brought about considerable improvement, and when this latter amount was fed from the beginning rapid growth and exceptionally sleek healthy appearance of the animals resulted. (E.g., mice 33 ♂, 51 ♂; chart I.) Forty per cent was therefore taken as the minimum requirement for growth and used as the standard for comparison in all work with the winter wheat.

DISTRIBUTION OF VITAMIN B IN MILLING PRODUCTS OF WINTER WHEAT. *Patent flour.* The experiments on patent flour agreed very well with the accepted belief that this product is very poor in vitamin B. When animals were fed a food containing 20 per cent of patent flour they lost

weight from the beginning and recovered only when offered a diet containing a product richer in vitamin. Forty-three per cent of patent flour gave no better results. (E.g., mice 41 ♀, 49 ♂; chart II.) This was the highest percentage that could be tried without disturbing the selected ratio of proteins and fats in the diet. An indirect method was therefore attempted for investigating the effects of larger quantities of flour. It had been found, as will be seen later, that 5 per cent of

CHART II



standard middlings was insufficient for growth, but 10 per cent was quite adequate. Two mice were given a food containing $6\frac{2}{3}$ per cent middlings and $33\frac{1}{3}$ per cent patent flour. This made a total of only 40 per cent wheat products and did not upset the protein and fat values of the food. However, it substituted $33\frac{1}{3}$ per cent of patent flour for one-third the minimum requirement of middlings. Under these conditions, if the 10 per cent middlings were surely the minimum for growth, any progress on this diet would indicate that $33\frac{1}{3}$ per cent

patent flour was equivalent in vitamin content to 3⅓ per cent middlings, or a diet of 100 per cent flour would contain just the minimum amount of vitamin for growth. The animals fed on this diet (e.g., mouse 74 ♀; chart II) grew, but not so well as normal mice, and their appearance was not particularly healthy. After 5 weeks the diet was changed to a food containing the same quantity of middlings without the patent flour. On this food growth continued at the same rate as before, without any noticeable change. The patent flour had evidently contributed no appreciable amount of vitamin B. If the food containing middlings and patent flour had given positive results, they would have indicated that the patent flour has only two-fifths the concentration of vitamin B that is present in the entire wheat, but the negative findings point to a still smaller concentration. There may be a small amount of vitamin B present in the patent flour, but it is certainly too insignificant to be of any practical value.

First clear: The quantity of vitamin B in a diet containing 20 per cent of this product was inadequate, even for maintainance, but a food containing 40 per cent furnished sufficient to promote nearly normal growth. (E.g., mice 37 ♀, 55 ♀; chart II.) The first clear represents 15 per cent of the entire wheat. If 40 per cent of the first clear were considered as potent as 40 per cent of entire wheat, which is entirely adequate, the first clear would contain about 15 per cent of the total vitamin B of the grain. Since 40 per cent of this product was nearly but not entirely as satisfactory as 40 per cent of entire wheat, it evidently contains less than 15 per cent of the vitamin. This result is about as accurate as the method employed permits.

Second clear. This was tested in the same way as the first clear. Twenty per cent of second clear in the diet was sufficient to maintain weight without growth. When this was increased to 40 per cent, normal growth was obtained. (E.g., mice 39 ♀, 57 ♀; chart II.) Second clear is therefore about equal to the entire wheat in its concentration of vitamin B, and since it makes up 5 per cent of the grain, it contains approximately 5 per cent of the total quantity of vitamin in the wheat.

Low grade flour. This was, in the series tested, the poorest grade as regards whiteness and freedom from bran of those flours intended for human consumption. It was, however, the richest of these in vitamin B. Twenty per cent in the diet was sufficient to bring about a good rate of growth, and since 25 per cent gave no better results, (e.g., mice 44 ♂, 60 ♀; chart II), 20 per cent was considered sufficient for growth. Being thus twice as rich in vitamin B as the entire wheat, and

making up 8 per cent of the wheat, this flour can be estimated to contain 16 per cent of the vitamin.

Standard middlings. This term is used to describe a cattle feed containing most of the germ, fine particles of bran and the softer portions of the endosperm which cling to these parts. It represents 10 per cent of the grain. A 5 per cent admixture of middlings was sufficient to ensure maintenance but not growth. Good growth was obtained, on the other hand, on a food containing 10 per cent, and no better results were gained with 20 per cent. (E.g., mice 48 ♀, 65 ♀, 54 ♀; chart II.) The concentration of vitamin B therein is consequently four times as great as that in the unmilled wheat, and the proportion milled into this part is 40 per cent of the whole supply.

Bran. The bran is the coarsest of the products studied, and contains the least amount of endosperm although small portions of the latter always adhere to the large bran flakes. Ten per cent of bran in the diet was not sufficient for growth. Approximately unchanged body weight was maintained on this food for over 4 weeks. The mice were restless and rough coated, and on the 33rd day one of them died. Animals receiving 20 per cent of bran showed good growth and were particularly sleek and healthy in appearance. (E.g., mice 46 ♀, 62 ♀; chart II.) The bran is evidently twice as rich in vitamin B as the entire wheat, and since it makes up 12 per cent of the whole, it contains about 24 per cent of the total vitamin.

DISTRIBUTION OF VITAMIN B IN HAND-DISSECTED PORTIONS OF KERNELS.

Marquis spring wheat: Bran. Commercial bran is in the form of large flakes and to these there adheres a small amount of the starchy endosperm. The actual quantity of the latter varies in different lots, but an appreciable minimum is unavoidable. Bran was therefore prepared as pure as possible by softening the grains for a time in water, cutting them in half lengthwise and scraping out the inside with a scalpel. This was done with the greatest care, so that the bran thus prepared had a pure brown appearance instead of being speckled with bits of white. Only one pair of mice was fed with this bran, owing to the great difficulty of preparing it in quantity. Calculations showed that if the bran contained all or nearly all of the vitamin B, 6 per cent of it in the diet should be sufficient for growth. A food containing 6 per cent of this hand-dissected bran was therefore fed and gave no evidence of containing any vitamin B. In view of the results with commercial bran which have already been outlined, but which were not obtained until later than these, it is not surprising that 6 per cent of purified bran

was insufficient, since 20 per cent of commercial bran was necessary for normal growth.

While this experiment showed that bran surely does not contain much of the vitamin, it threw no light on the question whether bran contains *any* of it; for larger percentages were not investigated. Experiments involving larger quantities became practically impossible due to the labor involved. The results obtained with 6 per cent of hand-dissected bran are, however, important in the following connection: They show that the bran present in a diet containing 40 per cent or less of wheat does not contain in itself sufficient vitamin B for growth; therefore if growth is induced by 40 per cent or less of any product contâining a proportion of bran not greater than that found in the unmilled grain, it must be attributed to something in the product other than the bran present.

Marquis spring wheat: Embryo. Weighed amounts of embryos prepared according to the method previously described were fed daily apart from the regular food. On the basis of 40 per cent, a very generous allowance in the case of entire spring wheat being necessary to furnish the requisite quota of vitamin B, 20 mgm. of embryos daily should have been entirely adequate for growth if the embryo contained all the vitamin as Voegtlin and Myers (1919) stated that it does.

One mouse was offered in successive periods 20, 40 and 80 mgm. daily of embryos, and finally 40 per cent of entire wheat. Although considerable growth was induced by the embryos, a fully normal condition did not result until the entire wheat was fed. Another mouse, which had been maintaining a good weight for some time on 40 mgm. of embryos daily, declined at once when this source of vitamin B was removed from the diet. Twenty and 40 mgm. of embryos, when fed daily to another pair of mice which were in bad condition from a diet poor in vitamin, caused only slight improvement. Apparently as small a quantity as 20 mgm. of embryos contained an appreciable amount of vitamin B, and as large a dose as 80 mgm. was not fully adequate for normal growth.

Marquis spring wheat: Endosperm. The grains from which the embryos had been removed were ground and used as a source of endosperm. A small part of the bran was sifted out with a wire sieve, but much of it remained. Judging from the experiments on bran, the edible quantity present in any of the foods described in this section could not possibly induce growth alone unless the endosperm also supplied vitamin. Allowing for the embryos and bran removed, 36

per cent of this deëmbryonated wheat was considered the equivalent of 40 per cent of entire wheat. A food containing 36 per cent of endosperm was therefore tested. Since some growth, but at a rate below normal was obtained with this diet, it is evident that the endosperm contains some vitamin B but not all of that present in the kernel. Since the same decision has been reached in regard to the embryo, viz., that it contains some but not all of the vitamin, it would seem that the essential substance is distributed more or less throughout the grain, and not concentrated in one part.

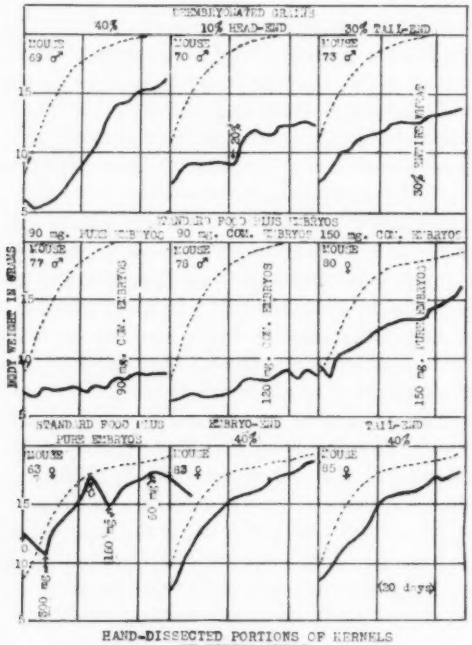
Osborne and Mendel (1919) found the concentration of vitamin B greater in the part of the endosperm next to the embryo than in the opposite end. Experiments were undertaken to repeat their investigation. Deëmbryonated grains were cut in two crosswise. One fraction consisted of about a fourth of the grain from the end nearest the embryo and the other of the remaining three-fourths from the opposite end. Each fraction was ground and a portion of the bran sifted out, as in the case of the undivided deëmbryonated grains. Nine per cent of the end next the embryo, or "head-end" as it is here designated for convenience, was taken as the equivalent of that part of the wheat in 40 per cent of the entire grain. Thirty-six per cent of the other or "tail-end" was similarly used. Both ends evidently contained some vitamin, for the mice grew much better than would have been possible without any. In the percentages tested neither food contained sufficient for normal growth, but the results tended on the whole to bear out Osborne and Mendel's conclusions that vitamin B is more concentrated in the end of the endosperm nearest the embryo.

Minnesota winter wheat. In view of the difficulty of preparing pure bran and of the available data for commercial bran milled from the same lot of wheat as that which was used in these experiments and which has already been discussed with the other milling products of this wheat, no further investigations on bran were attempted. A number of experiments was performed on the embryos and the deëmbryonated wheat, both prepared in the same way as the corresponding parts of the spring wheat except that none of the bran was sifted out after grinding.

To determine whether any large amount of vitamin was lost into the water in which the wheat was boiled to facilitate the removal of the embryos, mice were fed a diet containing 40 per cent of entire wheat which had been boiled and dried in the same manner as the wheat which was dissected. The results indicated that no large proportion of the vitamin B was lost from the wheat by boiling.

Minnesota winter wheat: Embryo. As in the case of the spring wheat it was calculated that 20 mgm. of embryos daily would represent the quantity of embryos in 40 per cent of entire wheat. Twenty, 40 and 60 mgm. respectively faily proved to be entirely inadequate for growth; 150 mgm. induced growth at a retarded rate, and 300 mgm. (fed to one mouse only) promoted normal growth. The minimum adequate

CHART III



HAND-DISSECTED PORTIONS OF KERNELS OF WINTER WHEAT

quantity probably lies somewhere between 150 and 300 mgm. per day. Several comparisons between hand-dissected and commercial embryos showed no appreciable difference in the concentration of vitamin B in the two products. (E.g., mice 77 ♂, 78 ♂, 80 ♀, 63 ♀; chart III.)

Minnesota winter wheat: Endosperm. Forty per cent of deëmbryonated winter wheat was tested and found to contain a nearly adequate supply of vitamin B. Ten or 20 per cent of "head-end," comprising about one-fourth of the grain, contained some vitamin B,

but not enough for continued growth. Thirty per cent of "tail-end," making up the remainder of the grain, promoted growth at a subnormal rate. Thirty per cent of entire wheat substituted for the "tail-end," served to keep the growth rate about the same. The "tail-end" of the wheat grain may therefore be considered to have the same concentration of vitamin B as the entire grain. In the case of this winter wheat there was no proof that the "head-end" was any richer in vitamin B than the "tail-end." (E.g., mice 69 ♂, 70 ♂, 73 ♂; chart III.)

If any doubts are entertained regarding the freedom of the "head-end," from bits of embryo in spite of the care used in dissection, there can surely be no question of contamination with embryo of the "tail-end," from which the entire embryo-end of the grain was cut away. The growth obtained with this part of the wheat is therefore conclusive proof that *vitamin B is not confined solely to the embryo.*

Further proof that not all, nor even the greater part of the vitamin is contained in the embryo of wheat was obtained by cutting grains in half (without previous boiling) and feeding the end with the embryo to one pair of mice and the opposite end to another pair. If the embryo contained a large portion of the vitamin, the mice receiving the end of the grain containing it would be expected to grow very much better than those receiving an equal quantity of the other end of the grain. Foods containing 40 per cent of the respective ends were tested. All the mice grew very closely to normal and were sleek and healthy in appearance. Between the two pairs no appreciable difference could be observed that could be interpreted to show differences in the vitamin content of the two ends of the grain. (E.g., mice 83 ♀, 85 ♀; chart III.) The quantity of vitamin B in the embryo was evidently not great enough to make the end of the grain in which it resided noticeably richer than the other end. Judged from the previous experiments with entire wheat, deëmbryonated wheat and "head-end" and "tail-end" of endosperm, these quantities should not greatly have exceeded the minimum for normal growth and thus obscured a possible effect of the embryo.

Discussion. The experiments with entire wheat described in the present paper have demonstrated that this grain contains an abundant supply of vitamin B. Mice grew well and maintained a healthy appearance on diets in which wheat was the sole source of water-soluble vitamin B.

Hart, Miller and McCollum (1916) and McCollum, Simmonds and Pitz (1916) claimed to have found in wheat a toxic substance demon-

strated by their experiments on swine and rats. No evidence for toxicity was found in the present investigation. If the percentage of wheat in the food was increased considerably above the minimum requirement no harmful results were obtained, but, on the contrary, even better growth and appearance than resulted from smaller amounts of wheat were frequently observed. All known dietary essentials were, of course, supplied.

The quantity of vitamin B in the two samples of wheat tested varied markedly, the spring wheat being two or three times as rich in this factor as the winter wheat. In investigating the distribution of vitamin in wheat it is therefore always necessary to determine the total concentration present in each variety to be tested.

In regard to the distribution of vitamin B in the different portions of the wheat kernel, the outstanding fact to be observed is that, contrary to many of the current statements, this dietary factor is not limited to any one part of the grain, but is widely dispersed throughout the entire kernel. It seems strange, considering the comparatively small percentage of the total vitamin B found in the germ and bran, that the patent flour is so very poor in this factor. This may probably be explained as follows: In the processes of milling the softer, more friable parts of the endosperm which are rich in vitamin, adhere to the embryo and bran as they are flaked off from the grain. Harder lumps of endosperm, poor in vitamin, are left behind and when sufficiently freed from germ and bran are themselves ground into fine white flour. This appears to be the most plausible explanation for the difference between the higher and lower grade flours.

An attempted estimate of the distribution of the vitamin B in milling products of Minnesota winter wheat may be summarized in tabular form as follows:

TABLE 1

PRODUCT	MINIMUM PERCENTAGE ADEQUATE IN DIET	CONCENTRATION OF VITAMIN B REFERRED TO WHOLE WHEAT	PERCENTAGE WHOLE WHEAT MILLED INTO EACH PART	PERCENTAGE VITAMIN B CONTAINED IN EACH PART
Whole wheat	40	1	100	100
Patent flour	(?)	0–0.10(?)	50	0–5
First clear	40(?)	1–0.67(?)	15	15–10
Second clear	40	1	5	5
Low grade	20	2	8	16
Middlings	10	4	10	40
Bran	20	2	12	24
Total			100	100

The distribution of the vitamin in the true structural parts, as distinguished from the commercial milling products, has not been quite so definitely worked out, but it can be calculated roughly. The minimum amount of embryos necessary for growth for mice is probably between 150 and 300 mgm. daily Accordingly the percentage of vitamin B in this part appears to be between 8 and 16 per cent of that in the entire kernel. The bran, according to the result obtained from the commercial product, contains approximately 24 per cent. Subtracting these percentages from 100, leaves 60 to 68 per cent of the vitamin in the endosperm—a much larger quantity than has been usually accredited to that part of the grain.

The current belief that all or most of the vitamin B is contained in the embryo is founded on its relatively great *concentration* therein which has been confirmed in the foregoing experiments. It has been demonstrated that the embryos contain a *concentration* of vitamin B four to eight times as great as that of the whole wheat, even though, due to the fact that they make up but 2 per cent of the grain, the absolute quantity contained in them is less than a sixth of the total amount present in the kernel. A gram of wheat embryos contains more vitamin B than a gram of any other portion of the berry; but in a gram of entire wheat only a small proportion of the vitamin is contained in the embryos.

As has been stated before, Osborne and Mendel (1919) succeeded in maintaining rats with pure embryos, but were unable to induce growth by any of the quantities tested. They interpreted these results as indicating that the growth factor and the antineuritic factor, commonly considered one substance and designated vitamin B, may be two separate entities, the former residing in the endosperm of wheat and the latter in the embryo. Other evidence against the identity of these factors has been presented by Mitchell (1919), Emmett and Luros (1920 and 1920a), Emmett and Stockholm (1920) and Funk and Dubin (1921). No confirmation of the separation of the two factors in the wheat kernel was obtained in the present investigation. When fed small quantities of embryos, mice lost weight and died; larger quantities maintained the animals for considerable periods; when the amount fed was great enough, actual growth was induced. In this respect the pure embryos did not differ materially from the commercial embryos or other wheat products.

In regard to the endosperm, Osborne and Mendel (1919) concluded the end next the embryo to be richer in vitamin B than the opposite end. The evidence in the present work for or against this view was not very conclusive.

SUMMARY AND CONCLUSIONS

The cereal grains and foods prepared from them constitute a large part of the diet of man and some of the domestic animals in all parts of the world. In this country and in most of Europe wheat occupies a preëminent position among the food cereals. Latterly the attention of students of nutrition has been directed not only to the long-familiar nutrients present in wheat but also to the more recently discovered food factors known as vitamins.

In view of the conflicting opinions entertained regarding the distribution of vitamin B in wheat and its products, further investigations of this question were undertaken. Foods containing various percentages of wheat and wheat derivatives and comparable to each other in their content of all known dietary essentials except vitamin B were fed to albino mice, and the rate of growth on these foods was compared with normal rates.

When the entire wheat kernel is thus used as the source of vitamin B in the diet, from 15 to 40 per cent of the cereal is required to insure growth at a normal rate. In comparison with many other food materials of which 2 to 10 per cent in the diet is sufficient to supply the requisite amount of vitamin B, wheat shows a relatively small content. Different varieties of wheat vary in this respect. A comparison of samples of Marquis spring wheat and Minnesota winter wheat showed a greater concentration of vitamin B in the former.

All the milling products from a single lot of winter wheat were studied and the approxmate concentration of vitamin B in each product was calculated. Patent flour contained no appreciable vitamin; first clear and second clear displayed about the same concentration as the unmilled grain; low grade flour and bran were about twice as rich; standard middlings (which included the portion containing most of the embryo) were four times as rich as the entire grain. These findings are physiologically significant in view of the widespread use of highly milled patent flours to furnish a liberal part of the food intake of man. It is also evident that the inclusion of milling products comparatively rich in vitamin B in the feeds of domestic animals represents a nutritive advantage to them.

Hand-dissected portions of grains, representing more nearly the true *structural* divisions of both spring and winter wheat were also investigated. Vitamin B was found in both embryo and endosperm. The *concentration* in the former was several times as great as that in the latter, but owing to the small percentage of the entire kernel represented

by the embryo, the actual absolute quantity of vitamin B contained thierein was not over a sixth of the total amount in the grain. No difference could be detected between hand-dissected and commercial embryos.

Finally, wheat grains were cut in half crosswise and equal quantities of the two ends were fed to different animals. The rate of growth induced by both ends was practically the same. The presence or absence of the embryo did not make enough difference in the vitamin content of the two portions to be apparent.

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THE EFFECT OF PANCREATIC EXTRACT (INSULIN) ON NORMAL RABBITS

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The successful demonstration of the presence in extracts of degenerated and fetal pancreas (1) of a substance capable of reducing the degree of hyperglycemia and of raising the carbohydrate tolerance of diabetic (depancreated) dogs, and the subsequent discovery that potent extracts may also be prepared from the adult gland, led to the question whether the blood sugar of normal animals would also be affected by the same substance. For both theoretical and practical reasons this question is of great importance; theoretically, because if the blood sugar of normal, as well as diabetic animals, should be affected it would indicate that the action of the active principle in the extract (insulin) is a fundamental one in the control of blood sugar; and practically, because it would place in our hands a ready method by which to determine the potency of the extracts and to investigate various modifications in the methods of their preparation.

Before we proceed to give the results of our investigation it may be well briefly to refer to some of the previous publications in which substances capable of lowering the blood sugar of normal and diabetic animals are discussed. The best-known of the substances is phlorhizin, the action of which was discovered in 1885 by v. Mering (2). The hypoglycemic action, in dogs at least, was also definitely shown by various investigators particularly by Minkowski (3). Not only does this drug lower the blood sugar of normal dogs but also of those rendered diabetic by removal of the pancreas (4), (5). The degree of hypoglycemia that can be caused by phlorhizin is however not very marked; indeed in some animals, such as the rabbit, it may cause a slight rise in blood sugar. Hypoglycemia is also a symptom of phosphorus poisoning and it becomes of extreme degree when phlorhizin is given to phosphorus-poisoned dogs (6). Underhill (7) found that decided

though variable degrees of hypoglycemia occurred in one to three days after the administration, subcutaneously to dogs, of 50 mgm. hydrazine sulphate per kilo body weight. No characteristic symptoms were observed except extreme weakness. Similar results were not so constantly obtained in rabbits. It was further observed that death occurred when dextrose was injected subcutaneously (5 grams per kgm.) into dogs treated two days previously with non-fatal doses of hydrazine. Further observations by the same worker (8) showed that hydrazine in the above dosage is capable of preventing glycosuria in depancreated dogs, the inhibiting effect lasting for between two and four days. Not only did glycosuria fail to appear at the usual time after pancreatectomy in hydrazine-treated dogs but the glycosuria caused by pancreatectomy in normal animals could be inhibited by subsequently injecting hydrazine. The blood sugar also failed to rise or did so only slightly when pancreatectomy was performed in dogs previously injected with hydrazine. Salts of uranium, etc., also cause hypoglycemia (cf. MacNider).

Interesting though these observations are from a scientific standpoint, the results offer nothing of practical value in the treatment of diabetes in man. In this connection the observations of Underhill (9) and his collaborators (10) and of Murlin (11) and Kramer (12) are important. Briefly stated, the work of the former group shows that administration of alkaline carbonates can bring about a decided reduction in the hyperglycemia and glycosuria caused by injections of adrenalin in rabbits, but has no effect on the percentage of blood sugar in normal rabbits. Underhill (13), however, found that marked reduction in the sugar elimination could be brought about in a severe case of diabetes in man by prolonged ingestion of large doses of sodium bicarbonate. Murlin and Kramer by observations on the respiratory quotient, found that alkali administration facilitates the combustion of sugar in the depancreated dog. Injections of sodium carbonate into the blood stream of such animals also caused lowering of the blood sugar. These results, taken along with the previously known fact that administration of acids lowers the tolerance for sugar, are interpreted as indicating that the regulation of the percentage of sugar in the blood is associated to a certain extent with changes in the acid-base equilibrium of the body (14). From the standpoint of the present investigation, the important point is that alkali administration can reduce the blood sugar and diminish the extent of glycosuria in certain forms of diabetes, both experimental and clinical.

Another interesting type of hypoglycemia is that which develops after removal of the liver from the circulation. One of us (J. J. R. M.) (15) in conjunction with R. G. Pearce has shown that the blood sugar rapidly declines in animals from which the liver has been removed, thus confirming those previously made by Bock and Hofmann, Pavy, etc. More recently Mann and Magath (16) have shown in similar experiments that when the percentage of the blood sugar falls to a certain level (0.06 or less), characteristic symptoms of muscular weakness and coma begin to develop, passing later into a final stage with convulsions. The symptoms usually appear in 5 to 8 hours after removal of the liver and that they are definitely related to the reduction in blood sugar is shown by the fact that they are instantly removed by the injection (intravenous?) of solutions of glucose, or even of galactose and maltose. The recovery may last some time and if the symptoms reappear they can again be removed by the injections. By repeated injections the animal may be kept alive for 15 to 30 hours.

The attention of several workers has recently been turned to a form of hypoglycemia which is possibly of much greater physiological significance than any hitherto studied, namely, that which develops following alimentary hyperglycemia, or even after the ingestion of non-carbohydrate foodstuffs. Folin and Berglund (17) have found, for example, that the percentage of blood sugar in man may fall as low as 0.054 to 0.058 immediately following the rise caused by ingestion of glucose. Maclean and Wesselow (18) have also recently investigated this form of hypoglycemia. This work recalls the observations made some years ago by Vosburg and Richards (19) on dogs and by Levie (20) on man in which it was found that hypoglycemia develops after the hyperglycemia due to adrenalin. A certain degree of it was also observed in rabbits following the hyperglycemia due to intravenous injections of glucose (Jacobson, Bang).

Finally, we must allude briefly to the researches which have more directly led up to the present, namely, those on the effects of pancreatic extracts. Recognizing, as previous investigators also did, that the failure to obtain extracts which could influence the metabolism of sugar might be due to destruction of the specific hormone by proteolytic enzymes, E. L. Scott (21), in 1912, used alcohol as the extracting agency, but the extracts did not decidedly lower the sugar excretion. He found, however, that watery extracts given intravenously did temporarily lower the D/N ratio. Murlin (loc. cit.) also observed reduction in the sugar excretion in diabetic dogs by injecting alkaline

extracts of pancreas and Kleiner (22) was able to bring about a definite lowering of the blood sugar in such animals by slow injections of unfiltered watery extracts of the gland. Paulesco (23) also briefly reports favorable results. The most definite and constant results on this aspect of the problem have been those recently published from this laboratory (24), (25), (26), and it is especially significant that the observations on diabetic dogs have been confirmed in the medical clinic of the University of Toronto by similar ones on several diabetic patients (27).

METHODS. In order to establish tolerable uniformity in the nutritive condition of the rabbits used in this investigation, the animals have been fed on a diet of oats and hay. The blood obtained from the marginal ear vein was allowed to drop into a small crucible containing a small amount of powdered oxalate (an excess must be avoided since it interferes with the precipitation of the proteins). When there was any difficulty in securing a free flow of blood, as in an almost moribund animal, we have found that a satisfactory flow could almost invariably be obtained by causing vasodilatation by the application of some xylol to the tip of the ear.

The sugar was determined in the blood samples by the Schaffer-Hartmann method which we have found to be extremely satisfactory. At the termination of the observations whenever possible the glycogen in the liver was determined by Pflüger's method using the Schaffer-Hartmann method to determine the sugar after suitable dilution of the hydrolyzed glycogen solution.

The usual procedure was to take a sample of normal blood and then inject the insulin in several places subcutaneously after which further samples of blood were taken at regular intervals. The animals were meanwhile kept under constant supervision in order to observe the time of onset of any symptoms.

RESULTS. During the course of several hours the blood sugar of a rabbit may vary somewhat, the first two or three samples being higher than those removed subsequently (cf. Bang). The following observation probably shows the maximum extent to which this may occur.

Rabbit (2.39 kgm.) fed oats and hay

10:15 Blood sugar 1.27 per cent
11:15 Blood sugar 1.12 per cent
12:15 Blood sugar 1.12 per cent
2:15 Blood sugar 1.04 per cent
3:15 Blood sugar 0.97 per cent
4:15 Blood sugar 0.94 per cent
5:15 Blood sugar 1.04 (animal struggling)

Since it is upon the behavior of the curve that the assay of various preparations of insulin depends it is obviously important that this spontaneous fall in blood sugar should be borne in mind. Usually, however, the fall with active extract is much more rapid and pronounced than is ever observed under normal conditions. The following are detailed protocols of typical experiments.

Experiment, February 9, 1.6 kgm.

11:15 a.m. Blood sugar 0.167 per cent.
11:30 a.m. Subcutaneous injection of 4 cc. insulin.
12:00 m. Blood sugar 0.137 per cent.
1:45 p.m. Blood sugar 0.065 per cent.
3:00 p.m. Rabbit lying on its side apparently more or less unconscious and with rapid shallow breathing of a periodic character. Conjunctival reflex sluggish, eyeballs protruding and pupil widely dilated. Mechanical stimulation of the skin caused convulsive movements in which the animal threw itself about violently and rolled over sideways in the same direction, with head retracted. Each seizure lasted 2 to 3 minutes. Rectal temperature 37°C. If left to itself convulsive seizures might supervene without apparent exciting cause. In these the animal was lying on its side with the head retracted and the limbs contracting and relaxing as in running. After each convulsion the animal seemed to be better and could hold his head up but in a few minutes it again became comatose, another convulsion being common in about 15 minutes.
3:50 p.m. Blood sugar 0.028 per cent.
5:30 p.m. 4.5 grams pure dextrose in 20 per cent solution injected in various places subcutaneously. Within 5 to 7 minutes the rabbit was perceptibly improved and soon sat up in the normal position and apparently became normal (jumped about the room, etc.). Kept near radiator over night.

February 10. Animal hyperexcitable, the jaw constantly moving as in chewing, but ran about in normal way and ate food.

February 13. Has been eating freely but is still decidedly hyperexcitable, the jaw still showing chewing movements. Shook head violently when lifted by the ears. Losing weight.

February 14. The same.

February 15. Animal still losing weight. At 3 p.m. fits similar to those of the 9th reappeared and these continued at about 15 minute intervals until 5 p.m. when 5 grams dextrose were given subcutaneously with the result that the animal immediately recovered.

February 16. The rabbit was found dead in the cage and on post-mortem examination the pancreas was observed to be greatly atrophied (possibly autolytic) and the subcutaneous tissues of the anterior abdominal wall (where no subcutaneous injections had been made) changed to a mass of muciginous material. At various places under the skin of the back where injections of sugar had been made there was discoloration and induration of the tissues but no trace of the mucin-like material.

Experiment, February 10, 1.6 kgm.

12:00 noon. 4.5 cc. insulin injected subcutaneously.
4:00 p.m. Symptoms like those described above. It was particularly noted that the general condition of the animal was improved immediately following each convulsive seizure. (These occurred about every 5 minutes.)
4:15 p.m. 5 grams dextrose subcutaneously. The animal quickly recovered and by
5:30 p.m. was apparently perfectly normal.

Experiment, April 24

8:10 a.m. Blood sugar 0.129 per cent; 5 cc. insulin given subcutaneously.
8:55 a.m. Blood sugar 0.077 per cent.
Animal in convulsions some time before 11:30.
11:40 a.m. Blood sugar 0.047 per cent; rectal temperature 37.1°C.
11:48 a.m. 2.5 cc. more insulin injected.
12:00 noon. Convulsions, rectal temperature 36.0°C.
12:10 p.m. Blood sugar 0.033 per cent rectal temperature 36.0°C.
12:15 p.m. Convulsions.
12:18 p.m. 5 grams dextrose in 40 cc. water injected in several places subcutaneously—rectal temperature 36.0°C.
12:23 p.m. Blood sugar 0.056 per cent; rectal temperature 36.5°C.; rabbit now sitting up and apparently normal.
12:43 p.m. Blood sugar 0.091 per cent; rectal temperature 36.5°C., rabbit normal.
1:05 p.m. Blood sugar 0.070 per cent; rectal temperature 37.6°C.; rabbit normal.
2:35 p.m. Blood sugar 0.043 per cent; rectal temperature 38.0°C.
3:35 p.m. Blood sugar 0.053; rectal temperature 37.6°C. Convulsions.
5:40 p.m. Blood sugar 0.024; rectal temperature 35.0°C. Convulsions.

The symptoms as described above are fairly characteristic, but slight variations may be observed in different rabbits. Very frequently the first symptom is one of hyperexcitability and of apparent fear so that the slightest stimulus, such as touching the animal, shaking the cage or floor or clapping the hands, causes the animal to rush wildly about in an incoördinate fashion with the vision apparently affected since objects in his path are not avoided. A still earlier symptom often observed consists in chewing movements as if the rabbit were hungry, and at this stage if food is available it will be voraciously eaten.

While there is no evidence of marked cardio-vascular disturbance in a typical convulsion (blood being usually readily obtained from the ear vein), this is sometimes present making it very difficult to obtain any blood. These cases are especially noticeable when impure preparations of insulin are used and the depression of blood pressure is probably due to peptone-like substances (histamine). A characteristic symptom

in this group of cases is paralysis of the hind limbs, due probably to anemia of the lower portion of the spinal cord.

In many rabbits typical convulsions may last only a few minutes after which the animal gradually recovers without sugar injections. Such animals have frequently been used several days later for testing the potency of extracts.

A most interesting *post-mortem* finding in animals that have died in a convulsive seizure and in those that have temporarily recovered as a result of sugar injections but died subsequently, is the extensive mucigi-nous change in the subcutaneous tissues of the abdominal wall. Sometimes this material forms a more or less circumscribed tumor and, particularly after sugar injections, a considerable amount of fluid may be present along with the mucin-like material. This fluid has strong reducing properties. Nowhere else in the body have we observed this peculiar change. It is not due to any local action of the insulin since this is always injected in the back.

We will now give in tabular form less detailed data of 100 rabbits injected with insulin. In Table 1 the percentage of blood sugar fell by 25 per cent or over within 2 hours of the injection.

There are observations on 32 animals in this group and convulsions were observed in eleven cases. The average percentage of blood sugar in these is 0.042. In computing this average there are several cases where the value is taken either somewhat before or after the 2-hour period but the deviation is about the same in either direction and the figure probably represents very closely the average percentage of blood sugar at which convulsions appear in rabbits.

In the next table 2, those observations are given in which the blood sugar fell between approximately 25 and 50 per cent within 2 hours of the injection.

There are observations on 29 animals in this group and convulsions were observed within 2 hours of giving insulin in only one case (ECN, March 16). In several other cases the percentage of blood sugar fell considerably below this level with no symptoms (FGB, April 5, April 13; JBC, February 23, April 19; CHB, Apri 20).

Table 3 gives the cases in which the blood sugar fell by at least 50 per cent during the period of observation.

In the twelve cases of table 3, convulsions occurred in three, the blood sugars being 0.03, 0.04 and 0.065 respectively. No convulsions were observed in six cases with approximately 0.05 per cent of sugar nor in one with 0.042 per cent.

TABLE I

Case in which blood sugar fell by approximately 50 per cent within 2 hours of injection

EXPERIMENT	BLOOD SUGAR		TIME AFTER EXTRACT	SYMPTOMS
	Before insulin	After insulin		
ECN. Feb. 16	0.111	0.045	35 min.	None
		0.0151	4½ hrs.	
ECN. Apl. 22	0.139	0.075	45 min.	None
ECN. Apl. 22	0.129	0.056	1 hr.	None
ECN. Apl. 25	0.132	0.067	35 min.	None
FGB. Apl. 6	0.145	0.060	1 hr. 30 min.	None
		0.049	3 hrs.	None
FGB. Apl. 10	0.151	0.071	2 hrs.	None
FGB. Apl. 12	0.157	0.047	2 hrs.	Convulsions in 2 hrs. after extract
		0.037	4 hrs.	
		0.165	23 hrs.	Recovered
FGB. Apl. 20	0.147	0.063	1½ hrs.	None
		0.069	4½ hrs.	
FGB. Apl. 20	0.139	0.077	1½ hrs.	None
		0.051	4½ hrs.	
FGB. Apl. 20	0.135	0.045	1 hr. 10 min.	None
FGB. Apl. 25	0.135	0.071	1 hr.	Convulsions in 2 hrs. after extract
		0.031	2 hrs.	
		0.153	9 hrs.	Died next day
JBC. Jan. 17	0.120	0.047	1¼ hrs.	None
JBC. Feb. 14		0.045	1½ hrs.	Convulsions
JBC. Feb. 16	0.111	0.045	35 min.	None
		0.151	4½ hrs.	
JBC. Feb. 28		0.040	1 hrs.	Convulsions
JBC. Mar. 3		0.025	35 min.	Coma-like symptoms, recovery with sugar
JBC. Mar. 17		0.060	2 hrs.	None
JBC. Mar. 25		0.060	1 hr.	Convulsions in 1½ hrs.
JBC. Mar. 28		0.040	1 hr.	Convulsions
JBC. Apl. 11		0.045	1½ hrs.	
JBC. Apl. 16	0.110	0.064	1 hr.	None
CHB. Apl. 5	0.137	0.064	2½ hrs.	Convulsions in 2 hrs.
CHB. Apl. 5	0.130	0.064	2 hrs.	None
CHB. Apl. 17	0.149	0.040	1½ hrs.	Convulsions
CHB. Apl. 18	0.127	0.037	2 hrs. 45 min.	Convulsions
CHB. Apl. 19	0.125	0.064	2 hrs.	None
CHB. Apl. 19	0.127	0.045	2½ hrs.	None
CHB. Apl. 19	0.127	0.065	2 hrs.	None
CHB. Apl. 19	0.115	0.069	1 hr.	None
CHB. Apl. 21	0.140	0.075	1 hr.	None
CHB. Apl. 24	0.132	0.065	1½ hrs.	None
CHB. Apl. 24	0.125	0.037	2 hrs.	Convulsions
		0.01	2¾ hrs.	Moribund

TABLE 2

Blood sugar fell between 25 and 50 per cent in 2 hours

EXPERIMENT	BLOOD SUGAR		TIME AFTER EXTRACT	SYMPTOMS
	Before insulin	After insulin		
ECN. Feb. 24	0.120	0.093	35 min.	None
		0.080	2 hrs.	
ECN. Mar. 16	0.108	0.063	35 min.	Convulsions
		0.067	1½ hrs.	
ECN. Mar. 17	0.135	0.114	30 min.	
		0.103	1¼ hrs.	
		0.083	2 hrs.	None
ECN. Mar. 29	0.117	0.080	35 min.	None
ECN. Mar. 27	0.137	0.082	30 min.	None
ECN. Mar. 30	0.124	0.089	35 min.	None
FGB. Apl. 5	0.123	0.090	1½ hrs.	
		0.069	2½ hrs.	
		0.037	3½ hrs.	None
		0.039	4½ hrs.	
FGB. Apl. 12	0.149	0.083	1½ hrs.	None
		0.095	3½ hrs.	
FGB. Apl. 13	0.111	0.069	1½ hrs.	None
		0.065	3½ hrs.	
FGB. Apl. 14	0.149	0.081	1½ hrs.	None
FGB. Apl. 18	0.135	0.071	1 hr. 25 min.	None
JBC. Dec. 22	0.126	0.087	2 hrs.	None
JBC. Dec. 16	0.119	0.101	½ hr.	None
		0.089	1¼ hr.	
JBC. Jan. 4	0.124	0.093	1 hr.	None
JBC. Jan. 10	0.117	0.073	1 hr.	None
		0.085	3 hrs.	
		0.085	6 hrs.	
JBC. Jan. 12	0.112	0.072	1 hr.	None
JBC. Jan. 16	0.125	0.075	1 hr.	None
JBC. Jan. 23	0.094	0.068	1 hr.	None
JBC. Feb. 23	0.094	0.046	3 hrs.	
		0.049	4 hrs.	
		0.057	5 hrs.	None
		0.058	6 hrs.	
		0.131	Next day	
JBC. Mar. 22	0.141	0.087	½ hr.	None
		0.078	1½ hrs.	
JBC. Mar. 28	0.120	0.095	40 min.	None
JBC. Mar. 31	0.130	0.088	1 hr.	None
JBC. Apl. 6	0.124	0.078	45 min.	

TABLE 2—*Concluded*

EXPERIMENT	BLOOD SUGAR		TIME AFTER EXTRACT	SYMPTOMS
	Before insulin	After insulin		
JBC. Apl. 7	0.142	0.092	50 min.	
		0.045	6 hrs.	Convulsions in 6 hrs.
JBC. Apl. 19		0.065	30 min.	None
JBC. Apl. 19		0.085	31 min.	None
		0.060	4 hrs.	
		0.053	5 hrs.	
CHB. Apl. 20	0.089	0.061	2 hrs.	None
CHB. Apl. 20	0.108	0.071	1½ hrs.	None
CHB. Apl. 23	0.150	0.100	1½ hrs.	None

TABLE 3

Cases not included in preceding tables in which the blood sugar fell by approximately 50 per cent during period of observation

EXPERIMENT	BLOOD SUGAR		TIME AFTER INSULIN	SYMPTOMS
	Before insulin	After insulin		
ECN. Apl. 24	0.095	0.073 (more insulin)	45 min.	
		0.057	1 hr. 40 min. after second injection	None
		0.052	2 hrs. 45 min. after second injection	
JBC. Apl. 19		0.085	½ hr.	
		0.060	4 hrs.	None
		0.053	5 hrs.	
CHB. Apl. 7	0.135	0.051	3 hrs.	None
CHB. Apl. 6	0.130	0.03	8 hrs.	Convulsions at 7 hrs.
CHB. Apl. 13	0.111	0.065	3½ hrs.	None
CHB. Apl. 13	0.173	0.081	3 hrs.	None
CHB. Apl. 19	0.113	0.050	3 hrs.	None
CHB. Apl. 21	0.109	0.053	4 hrs.	None
CHB. Apl. 3	0.119	0.083	4 hrs.	None
CHB. Apl. 17	0.165	0.04	3 hrs.	Convulsions
JBC. Apl. 19		0.065	2½ hrs.	Convulsions (5 gm. dextrose)
		0.030	19 hrs.	Convulsions
JBC. Apl. 19		0.042	3 hrs.	None

Taking the three tables together there are observations on 73 animals. The blood sugar fell below 0.045 in thirteen and of these convulsions were present in eleven. Of the two cases with blood sugar below 0.045 not showing convulsions, one (JBC, April 19) gave 0.042 in 3 hours, the other (FGB, April 5) 0.037 in ½ hours and 0.039 in 4½ hours. There are nine cases with blood sugars between 0.045 and 0.050 of which convulsions were observed in three. The highest percentage at which convulsions were observed was 0.064[1] (CHB, April 5) 0.063[2] (ECN, March 16) and 0.065 (JBC, April 19). The lowest percentage at which there were no symptoms as already mentioned was 0.037 (FGB, April 5).

The foregoing analysis shows that the average computed, from the results of table 1, as that at which convulsions most commonly appear, namely 0.042, must be very close to the correct one.

For purposes of physiological assay of insulin we consider that the most satisfactory basis at present is the number of cubic centimeters which lowers the percentage of blood sugar in normal rabbits to 0.045 in from 2 to 4 hours. There are several advantages in using this standard, among which the following may be mentioned.

1. Since 0.045 per cent is the level of blood sugar at which definite convulsions supervene one can tell in a general way from the premonitory symptoms of the injected animal (evidence of hunger and thirst, hyperexcitability and evidence of fear) whether or not this level is likely to be reached.

2. The effect of insulin in reducing the blood sugar of a diabetic (depancreated) dog is considerably more marked than one would expect from the effect on the normal rabbit. Just as a certain dose of an antipyretic may cause a marked lowering of hyperpyrexia in fever, but have only a slight effect on the normal temperature, so will insulin reduce the blood sugar relatively much more in hyperglycemic than in normal animals. This fact is illustrated in the following observation. Ten cubic centimeters of insulin lowered the percentage of blood sugar in a rabbit from 0.135 to 0.071 in 1½ hours; whereas 20 cc. given to a depancreated dog weighing 11 kgm. lowered the sugar from 0.375 to 0.030 in 19 hours. The rabbit weighed about 2 kgm. so that on a basis of body weight the dog received only about one-third the dose of the rabbit.

[1] Both of the rabbits from which these results were obtained were injected with crude extracts, containing much protein, and it is probable that the symptoms were of the paralytic type referred to on p. 167.

[2] The insulin used in this animal was evidently very potent since the blood sugar was 0.03 19 hours after it was given, dextrose being meanwhile administered. It is therefore possible that the blood sugar was really below the figure given when the convulsions occurred.

3. One unit as defined above has been used as the basis for use on man with sufficient frequency to warrant the assertion that while this dose markedly lowers the degree of hyperglycemia, it never causes any alarming symptoms, although the administration may be followed in an hour or so by certain subjective symptoms, such as hunger.

When the blood sugar does actually reach 0.045 per cent there is then no difficulty in stating the dosage of the preparation with tolerable accuracy. When it fails to reach this level however the assay is more or less unsatisfactory. There are several factors to be considered in this connection, the most important of which are:

1. The rate of physiological action of the insulin. There is no doubt that this varies among different preparations. It is decidedly slower in impure preparations than in those that give only faint protein reactions. With the former, the blood sugar may in 4 hours not yet have reached to its lowest level so that the preparation is assayed at too low a value; with the latter, the lowest point may have been reached before the 4 hours so that the assay is too high. At present we know of no method to remove these sources of error but we recommend, when the sugar is not lowered below 0.065 in 4 hours, that another specimen of blood should be taken in 5 or 6 hours and if the hypoglycemia is more pronounced the extent to which the action is delayed should be stated in the assay of the preparation. If, on the other hand, the blood sugar never reaches below 0.065 per cent with an injection of more than 4 cc. the preparation must be considered unsatisfactory. When the sugar falls to between 0.055 and 0.065 we arbitrarily label it a half rabbit dose and that this is a comparatively safe expedient is evidenced in the results of table 4 by the fact that there is only one case marked half a dose in which convulsions afterwards developed.

2. The same dose of the same preparation of insulin may not have the same effect on different rabbits. This is illustrated in the following results:

EXPERIMENT	AMOUNT INJECTED	BLOOD SUGAR		TIME AFTER INJECTION	SYMPTOMS
		Before	After		
	cc.	*per cent*	*per cent*	*hours*	
CHB. June 12..		0.14	0.09		
	2		0.08	2	None
June 12..	2	0.11	0.11	2	None
June 14..	2	0.13	0.06	2	None
June 14..	2	0.185	0.180	2	None
June 20..	2	0.12	0.065	2	Convulsions at 10 hours
June 20..	2	0.105	0.040	2	Convulsions at 3 hours

We have chosen observations which probably give the extreme degrees of variability likely to be met with, but the failure, as in the

TABLE 4

DATE	BLOOD SUGAR		AMOUNT	TIME AFTER INSULIN	SYMPTOMS	ASSAY (RABBIT DOSES)
	Before insulin	After insulin				
			cc.	*hours*		
June 5	0.148	0.038	4	4	Violent convulsions	1 plus
June 12	0.12	0.040	4	3	Violent convulsions	1 plus
June 14	0.13	0.062	2	2	None	½ plus
June 13	0.122	0.040	3½	2½	Convulsions	1 plus
June 13	0.125	0.040	3	2½	Convulsions	1 plus
June 15	0.146	0.054	2½	2	None	1
June 15	0.115	0.065	2½	2	None	½
June 15	0.155	0.062	3	2	None	½
June 15	0.120	0.066	2	2	None	½
June 16	0.130	0.055	2	2¼	None	½
June 19	0.130	0.060	2	2¼	Convulsions later	½
June 19	0.180	0.040	2	1¾	Convulsions	1 plus
June 19	0.140	0.060	2	2	None	½
June 19	0.120	0.060	2	2	Convulsions later	½
June 20	0.120	0.065	2	2	Convulsions later	½
June 20	0.130	0.065	1	1½	None	½
June 20	0.105	0.04	2	2	Convulsions	1 plus
May 8	0.135	0.056	2	1½	None	½
May 16	0.135	0.045	2½	2¼	Convulsions	1
May 16	0.120	0.045	1½	2¾	Convulsions	1
May 18	0.140	0.045	3	2½	Convulsions	1
May 1	0.11	0.045	1	2	Convulsions	1
May 10	0.11	0.062	3	1¼	None	½
May 2	0.165	0.045	10	2	Convulsions	1
May 3	0.116	0.062	2½	4	None	½
May 3	0.138	0.056	5	1	None	½
May 2	0.141	0.042	1½	4	Violent convulsions	1 plus

experiments of June 12 and 14, of preparations that were of considerable potency in one animal not to cause any hypoglycemia in others is obviously unsatisfactory. We are engaged in seeking for a more

dependable method of assay but at present must fall back on the above as the only one available.

In the following table 4, are given some of our most recent results (not included in the preceding tables) in which the above principles of assay are followed.

Further details will be given in a subsequent paper regarding the influence of injections of various sugars and related substances on the convulsions. For the present it is important to note that in practically every case in which we have injected dextrose (4 grams in 20 per cent solution) within half an hour of the first appearance of definite convulsive seizures, the animals have recovered as described in the experiment on p. 166. Even when the convulsions have been allowed to go on until the animal is evidently almost moribund dextrose injections usually have a most marked effect. For example, we have on several occasions succeeded in restoring to a tolerably normal condition an animal in which the breathing, after being extremely rapid or periodic in type, had at last ceased. Injections of equal quantities of saline solution have no effect. Frequently animals that have been restored to normal by dextrose pass a second time into convulsions which may again be antidoted by dextrose.

CONCLUSIONS

1. Purified alcoholic extracts of pancreas, for which we suggest the name *insulin*, when injected subcutaneously into normal rabbits cause the percentage of sugar in the blood to fall within a few hours.

2. As a tentative basis for the physiological assay of insulin we consider as one unit the number of cubic centimeters which causes the blood sugar of normal rabbits to fall to 0.045 per cent within 4 hours. This dose is decidedly active in lowering the blood sugar in diabetic patients.

3. As the blood sugar falls, as a result of insulin injections, the rabbit exhibits highly characteristic symptoms and earliest of which are signs of hunger and thirst, hyperexcitability and apparent fear.

4. The animal may recover from these earlier symptoms but frequently, with active preparations, the hyperexcitability becomes extreme and clonic convulsive seizures involving the entire body and lasting for several minutes supervene. Between the convulsive seizures the animal is lying on its side in a more or less comatose condition with shallow, rapid and frequently periodic breathing.

5. In the great majority of cases exhibiting convulsions the blood sugar has been found to be about 0.045 per cent.

6. Subcutaneous injections of dextrose solutions antidote the convulsions and other symptoms, so that the animal in a few minutes becomes restored to a tolerably normal condition. Similar symptoms may again develop but they also can be antidoted by dextrose.

7. In animals that die as a result of the symptoms, a peculiar mucigenous degeneration of the subcutaneous tissues of the abdominal wall is very commonly observed.

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EFFECT OF INSULIN (PANCREATIC EXTRACT) ON THE SUGAR CONSUMPTION OF THE ISOLATED SURVIVING RABBIT HEART

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It has been shown that insulin (1) (an alcoholic pancreatic extract prepared as described elsewhere) enables the diabetic animal and man (2) to metabolize sugar; also that in the case of the diabetic dog it causes a building up of glycogen (3), and that it reduces the blood sugar level in normal and diabetic animals (4). On the other hand it has been found that insulin does not influence the glycolytic power of blood *in vitro* or the rate of post-mortem glycogenolysis in the liver (4a). The cause of its hypoglycemic influence is therefore apparently resident in the active tissues and it becomes of interest to see whether it can alter the rate of sugar consumption by the surviving mammalian heart. It is with this aspect of the problem of the locus of action of insulin that the present paper is concerned.

It is scarcely necessary to review in detail the results of the numerous observations that have from time to time been made on the rate at which reducing substance disappears from nutrient fluid that has been repeatedly perfused through the surviving heart. The following results for the rabbit heart are, however, of importance for the interpretation of those of the present investigation. Locke and Rosenheim (5) in 4 experiments found an average consumption of 1.5 mgm. per gram heart per hour. Maclean and Smedley (6) in 4 experiments an average of 0.5 to 1.0 mgm.; Mansfeld (7) in 25 experiments 2.2 mgm. and Underhill and Prince (8), 0.7 to 1.6 mgm. Camis (9), on the other hand, in 20 experiments did not find any consumption of sugar, whereas Gayda (10) in 10 experiments found an average of 7.1 mgm.

Various investigators have studied the effects of pancreatic extracts on the rate of consumption of sugar by the isolated heart. Thus, Maclean and Smedley (loc. cit.) using diabetic dog and cat hearts found

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that the addition of pancreatic extract increased the sugar consumption in some cases up to normal and, as is well known, Knowlton and Starling (11) thought that the weakened sugar-consuming powers of the surviving heart of diabetic dogs could be greatly increased by the addition of pancreatic extract to the perfusion fluid. In a later paper however, Patterson and Starling (12) showed that the sugar consumption of the diabetic dog's heart is the same as that of a normal dog's heart. The most important of the recent work is that of A. H. Clarke (13). Working with dogs, this investigator found that the perfused pancreas consumed no sugar as judged by changes in the perfusion fluid but that the amount consumed by the heart was very decidedly increased when the nutrient fluid was first of all perfused through the pancreas and then through the heart, or when the two were perfused simultaneously. As a result of Clarke's investigations there can be little doubt that the pancreas delivers into the fluid perfused through it something that accelerates sugar consumption by the heart of the same animal.

METHODS. The principle of the method used to perfuse the heart was that of Locke (loc. cit). The animal was killed by a blow on the head, then quickly bled and the heart removed and placed in warm Locke's fluid. After being freed from fat, pericardium, etc., it was attached to the cannula of the apparatus and care was taken to ensure a constant temperature and thorough oxygenation of the perfusion fluid. This contained 0.2 per cent dextrose and was made up fresh for each experiment with hard glass-distilled spring water; pH was adjusted to 7.2. The apparatus was thoroughly sterilized before each experiment.

Locke (loc. cit.) has shown that such a fluid when oxygenated can be recirculated through the apparatus for 9 hours without glycolysis and that the perfusate, after a heart perfusion, can be incubated at 36°C. with toluol for 44 hours without glycolysis occurring.

Starling also states that if sterilization is carried out thoroughly, glycolysis due to bacterial action is negligible under 4 hours.

Records were taken every 15 minutes of temperature, heart rate and rate of flow in drops per minute. Sugar estimations were made every half-hour on 1 cc. samples, using the Schaffer-Hartmann (14) method. Most of the experiments lasted 4 hours. The first 100 cc. of perfusate were always discarded to get rid of traces of blood. In most cases the glycogen content of the heart was determined by the Pflüger (15) method at the end of the experiment.

The perfusate was hydrolyzed at the end of the experiment in two cases and showed no increase in the amount of reducing sugar. In estimating the sugar consumption in each observation, the loss of fluid by evaporation and removal of samples was taken into account by measuring the quantity left at the end of the experiment. The perfusion fluid measured 150 cc. to start with in most of the experiments.

RESULTS: *A. Cases without insulin.* Because of lack of uniformity in the results obtained by previous workers, it was considered necessary for us to collect data of our own for the normal heart beating under conditions that were precisely similar to those of the heart treated with insulin. We believe that the lack of uniformity of the results by previous workers is to be accounted for by the varying conditions of perfusion, particularly the chemical purity of the water and of the salts used in the preparation of Locke's solution, and the proper regulation of the pH. For comparison of the sugar consumption of different hearts it is obviously important that all these details be standardized. The details of a typical observation on the normal heart are given in table 1, from which it will be observed that the rate of consumption remains tolerably constant for about $2\frac{1}{2}$ hours, after which it diminishes at the same time as the flow of perfusion fluid becomes reduced. There were in all twelve observations of this type and the results are shown in graphic form in figure 1. The sugar consumption in milligrams per gram of heart per hour is shown in the white columns and the average blood flow (expressed as a percentage of the initial flow), the percentage of glycogen and the pH in the other, variously shaded columns. The average sugar consumption for all hearts is 0.87 mgm. per gram heart per hour which agrees almost exactly with the results of Maclean and Smedley, and Underhill and Prince. An exceptionally low rate of sugar consumption was found in one heart (no. XX) where it was associated with an unusually high percentage of glycogen. If we leave this result out, the average for the remaining hearts is 0.97 with a maximum of 1.5 and a minimum of 0.4 mgm. per gram muscle per hour. The maximum consumption occurred in a heart (no. XII) containing 0.2 per cent glycogen but it is impossible to make out any direct relationship between these two values in the remaining observations. Nor is there any evident relationship between pH or rate of flow and the sugar consumption. The glycogen content of this series varied from 0.143 to 0.995 per cent, with an average of 0.292 per cent. Leaving out the unusually high figure 0.995 in case XX, the average is 0.207 per cent.

TABLE 1

Protocol of experiment without extract

Rabbit XII. March 10, 1922. 250 cc. fluid. Perfusion commenced at 9:50. First 100 cc. discarded

TIME	SAMPLE	PER CENT SUGAR	pH	TEMPERATURE	RATE OF HEART BEATS PER MINUTE	FLOW IN DROPS PER MINUTE	REMARKS
10:00	1	0.185	7.3	38.0	128	130	Fair beat
10:15				38.0	128	128	
10:30	2	0.180		36.0	120	120	Foaming
10:45				37.5	120	68	
11:00	3	0.178		37.0	120	120	Better beat
11:15				37.0	120	124	
11:30	4	0.174		36.5	112	120	
11:45				38.0	112	110	
12:00	5	0.164		38.5	108	60	
12:15				37.5	198	80	
12:30	6	0.161		37.n	96	52	Fair beat
12:45				37.0	96	50	
1:00	7	0.162		37.5	90	44	
1:15				38.0	90	49	
1:30	8	0.156		38.0	94	48	Weakening
1:45				38.5	100	54	
2:00	9	0.149		38.5	120	52	
2:15				37.0	87	48	
2:30	10	0.148		37.5	92	48	

125 cc. fluid at end.

Sugar consumption = 1.5 mgm. per gram per hour.

Glycogen = 0.203 per cent.

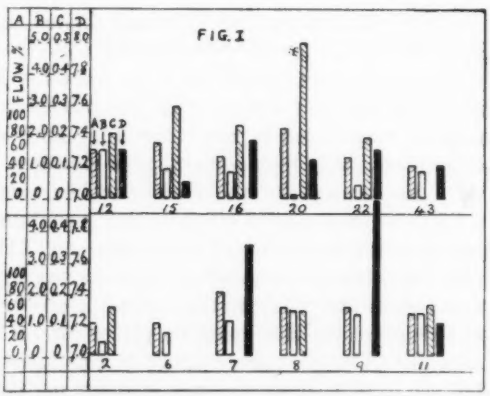

Fig. 1. Without insulin. *A*, Average flow as per cent of initial flow. *B*, Glucose used in milligrams per gram per hour. *C*, Per cent glycogen. *D*, pH. *, 0.995 per cent glycogen.

B. Cases with insulin. There were twenty observations in this group. The insulin was prepared in the Connaught Laboratories of the University of Toronto according to the methods worked out by F. G. Banting, C. H. Best, J. B. Collip and J. J. R. Macleod (16). Since it appeared that the traces of tricresol usually added as a preservative had an effect on the heart beat, only fresh preparations containing no preservative were used. They were not all of the same degree of purity, some containing larger traces of peptone-like material than others. The potency of the various extracts, as judged by their effects on the blood sugar percentage of normal rabbits was not uniform but in the later observations a preliminary test was usually made so as to control this factor.

The insulin was used in several ways:

a. It was added to the perfusion fluid and the pH adjusted before the experiment. This method was used in the earlier experiments but was not found to be entirely satisfactory.

b. It was added to 10 to 20 cc. perfusate removed about one hour after the beginning of the experiment; the pH was then adjusted and the fluid replaced in the apparatus.

c. Minute quantities, 0.1 to 0.25 cc. insulin were added to the perfusate every 15 minutes. This method was adopted in the later experiments and was found to be most satisfactory as it kept the pH at about 7.2, instead of increasing as it otherwise tended to do.

In several of the later experiments a dose of insulin was given subcutaneously 20 to 60 minutes before killing the animal and perfusing the heart.

The results of this series are shown graphically in figure 2. In the earlier experiments (13 to 27) and in experiments 32, 35, 36 and 37 the insulin was either of unknown strength or was found to be very weak. The sugar consumption of this group varied from 0 to 4.11 mgm. with an average of 1.9 mgm. The glycogen content of this series varied from 0.117 to 0.397 per cent with an average of 0.215 per cent. In 8 of the later experiments where the insulin was of proven potency, free from protein and Berkefelded, the consumption was as is given in table 2.

Kymographic tracings were taken of the heart in several experiments with the object of detecting any pharmacological action of insulin but beyond a slight improvement in flow no definite effect was observed. The rate of flow and the pH were satisfactorily similar in the two sets of observations.

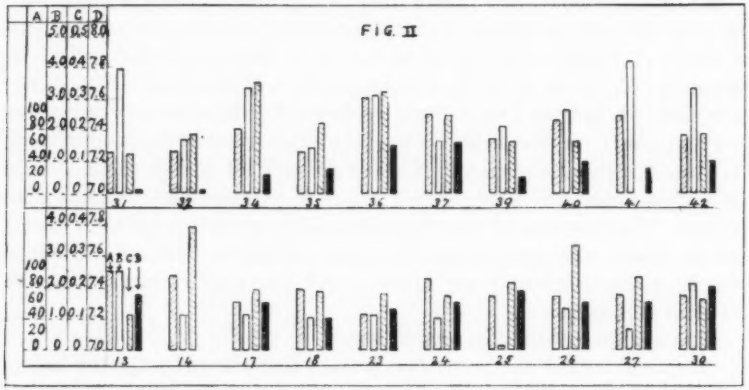

Fig. 2. Without insulin. *A*, Average flow as per cent of initial flow. *B*, Glucose used in milligrams per gram per hour. *C*, Per cent glycogen. *D*, pH.

TABLE 2

CASE	SUGAR CONSUMPTION PER GRAM HEART PER HOUR	GLYCOGEN
	mgm.	*per cent*
xxx	2.16	0.160
xxxi	3.90	0.120
xxxiv	3.30	0.350
xxxvi	3.06	0.311
xxxix	2.06	0.153
xl	2.58	0.155
xli	4.11	lost
xlii	3.29	0.179
Average.....	3.06	0.204

DISCUSSION

If we take the eight experiments in which there is no doubt that highly potent insulin was used it is clear that the consumption of sugar as compared with that of untreated hearts was greatly increased. In five cases (31, 34, 36, 41 and 42) this increase amounted to more than twice the maximum observed in the series of twelve hearts perfused without insulin and in two of the cases it was over four times the average for the normal hearts. The rate of flow diminished during each experiment to about the same extent in both series of observations

and, with one exception, the glycogen content was similar. Since there was no evidence, in two observations, that any of the reducing sugar that disappeared had become converted into a non-reducing form in the perfusion fluid and since it is certain that there could have been very little, if any, bacterial decomposition of sugar during the time of the observations, the conclusion can be drawn that insulin decidedly increases the power of the isolated heart to remove reducing sugar from the fluid perfused through it. As to whether the power to metabolize the sugar completely is altered cannot be said without investigation of the respiratory quotient and this we hope to undertake in heart-lung preparations in the near future. Since it has been shown in this laboratory that R.Q. is raised in depancreated animals when insulin is given along with sugar (16), the investigation referred to will be of great interest. Of the three ways by which reducing sugar in the perfusion fluid could be disposed of in the heart, namely, by polymerization into glycogen, by a partial splitting up into smaller molecules without complete oxidation and by complete oxidation, we have so far only collected evidence regarding the possibility of polymerization. This evidence is obtained by comparison of the glycogen content of the two series of hearts and as far as it goes does not indicate that such a process had occurred, the average percentage of glycogen in the normal heart being 0.207 and in the treated hearts 0.204. The considerable degree of variability in the different hearts of both series, however, makes these averages somewhat unreliable.

CONCLUSIONS

The average sugar consumption of the isolated rabbit heart perfused with Locke's solution was found to be 0.87 mgm. per gram per hour. When insulin of proved potency, as tested by its ability to lower the blood sugar in normal rabbits, was added to the perfusion fluid, the average sugar consumption rose to 3.06 mgm. per gram per hour.

The average glycogen content of the treated and untreated hearts was practically the same.

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